The interaction between hydrogenase activity and carbon metabolism in nitrogen-fixing legume root nodules

Thesis

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The interaction between hydrogenase activity and carbon metabolism in nitrogen-fixing legume root nodules

by

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THE INTERACTION BETWEEN HYDROGENASE ACTIVITY AND CARBON METABOLISM IN NITROGEN-FIXING LEGUME ROOT NODULES.

ABSTRACT

Rhizobium and Bradyrhizobium species fix atmospheric nitrogen (N_2) in symbiotic association with legumes. The endosymbiotic bacteroid-forms contain the nitrogenase enzyme complex which catalyzes the reduction of N_2 to produce ammonia (NH_3) using energy derived from the respiration of photosynthate supplied by the host plant. Hydrogen is also evolved as a by-product of the nitrogenase reaction and at least 25% of the energy is wasted through H_2 evolution. However, some strains of rhizobia possess a hydrogen oxidising enzyme called 'uptake hydrogenase' (Hup), that can recycle the H_2 evolved as a by-product of N_2 fixation and regenerate energy in the form of ATP.

This dissertation considers three topics relating to the physiology of symbiotic N_2 fixation (i) what is the energy cost of symbiotic N_2 fixation for the whole plant, and how significant are the losses associated with H_2 evolution ? (ii) what factors limit the rate and efficiency of symbiotic N_2 fixation ? (iii) Does the hydrogen recycling (uptake hydrogenase) system confer any advantages to the bioenergetics of symbiotic N_2 fixation ? The scientific literature in each of these areas is reviewed, and some suggestions made concerning future experiments which might help to investigate the contribution of the uptake hydrogenase system to the energetics of symbiotic N_2 fixation.

Rhizobia are obligate aerobes but the nitrogenase system is very sensitive to oxygen damage. Consequently, the root nodule is maintained as a microaerobic environment for the N_2 fixing bacteroids. The O_2 supply to the centre of nodules appears to be tightly regulated and in some cases the availability of O_2 may limit the rate of respiration and hence the energy available for N_2 fixation. In addition, the carbon supply to bacteroids is a major factor limiting the activity of root nodules. Environmental factors such as fixed nitrogen in the soil, other mineral nutrients, temperature and moisture, etc, also affect nodulation and N_2 fixation by rhizobia.

Experiments conducted with Hup^+ and Hup^- isogenic strains show that the Hup system is beneficial in the soybean-Bradyrhizobium symbiosis. In the pea-Rhizobium symbiosis the beneficial role of uptake hydrogenase is not yet established and more experiments are needed to determine the role of Hup in this symbiosis. Four future experiments are suggested.

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Chapter 1

BIOLOGICAL NITROGEN FIXATION AND THE LEGUME-*RHIZOBIUM* SYMBIOSIS

1.1. Introduction:

The availability of nitrogen in a utilizable form is a prerequisite for plant growth. Atmospheric nitrogen (N_2) can enter biological systems only when it has been "fixed" or combined with certain other elements such as hydrogen or oxygen. Atmospheric nitrogen can be fixed either industrially or biologically by microorganisms or through other natural processes such as lightning. Today the fixation can be accomplished industrially through the manufacture of ammonia from hydrogen and atmospheric nitrogen, but the bulk of all fixed nitrogen is of biological origin. In nature the process of nitrogen fixation is carried out solely by some prokaryotic organisms and is called "biological nitrogen fixation". Some of the nitrogen-fixing organisms (diazotrophs) are listed in Table 1.1. Organisms that can fix N_2 in the absence of other living organisms are termed free-living N_2 fixers. Such organisms include many aerobic, anaerobic and facultative anaerobic bacteria and also cyanobacteria. Some bacteria and cyanobacteria fix nitrogen in symbiotic association with some higher plants. In symbiotic nitrogen fixation the combined nitrogen is delivered directly into eukaryotic plants by the nitrogen-fixing bacteria.

Table 1.1 Biological nitrogen fixing organisms (adapted from Postgate and Hill, 1979)

Free-living	Aerobic		Azotobacter, Azomonas, Azotococcus, Beijerinckia, Derxia, Rhizobium, Xanthobacter, Methane oxidising bacteria, Azospirillum, Aquaspirillum, Thiobacillus ferrooxidans, Cyanobacteria(P)
	Anaerobic		Clostridium, Desulfovibrio, Desulfotomaculatum, Chlorobium (P), Chromatium(P)
	Facultative anaerobic		Klebsiella, Enterobacter, Citrobacter, Erwinia herbicola, Bacillus, Rhodospirillum(P), Rhodopseudomonas(P), Rhodomicrobium(P)
Symbiotic	Root nodules	Legumes + Rhizobium	
		Non-legumes+ Frankia	
	Azolla, Lichens + Cyanobacteria (P)		

(P) indicates photosynthetic microbes.

Not all species of the genera listed above are capable of nitrogen fixation; for example in Bacillus, Klebsiella and Clostridium only a relatively small number of species and strains have been shown to fix N_2.

1.2. Biological N_2 fixation and the nitrogen cycle:

In natural environments a delicate balance is established between the rate of plant growth and the supply of available soil nitrogen. The amount of nitrogen available in the soil is controlled by the rate of accumulation of nitrogen through N_2 fixation, and the processes of ammonification and nitrification of organic nitrogen and by the rate of depletion of fixed nitrogen via leaching and denitrification. A simplified version of the nitrogen cycle is presented in Fig 1.1. Biological nitrogen fixation contributes approximately 69% of the total nitrogen fixed worldwide, whereas the industrial synthesis of nitrogenous fertilizers accounts for only 15% (Burns and Hardy, 1975).

1.3. Legume-Rhizobium symbiosis:

The best known among the nitrogen-fixing symbiotic associations is the one between the plants called legumes and various bacteria of the genus Rhizobium. Legume crops are normally self-sufficient in fixed nitrogen because of their symbiotic associations with Rhizobium whereas most other agricultural crops such as cereals require manure or fertilizer as a source of fixed nitrogen. However, the industrial production of nitrogenous fertilizer by the Haber-Bosch process is an energy intensive process. Because of recent shortages and the rapid depletion of fossil fuel supplies with subsequent increased cost, the production of nitrogenous fertilizer has become more and more expensive. Moreover, many of the grain legume crops are very rich sources of protein for animal or human

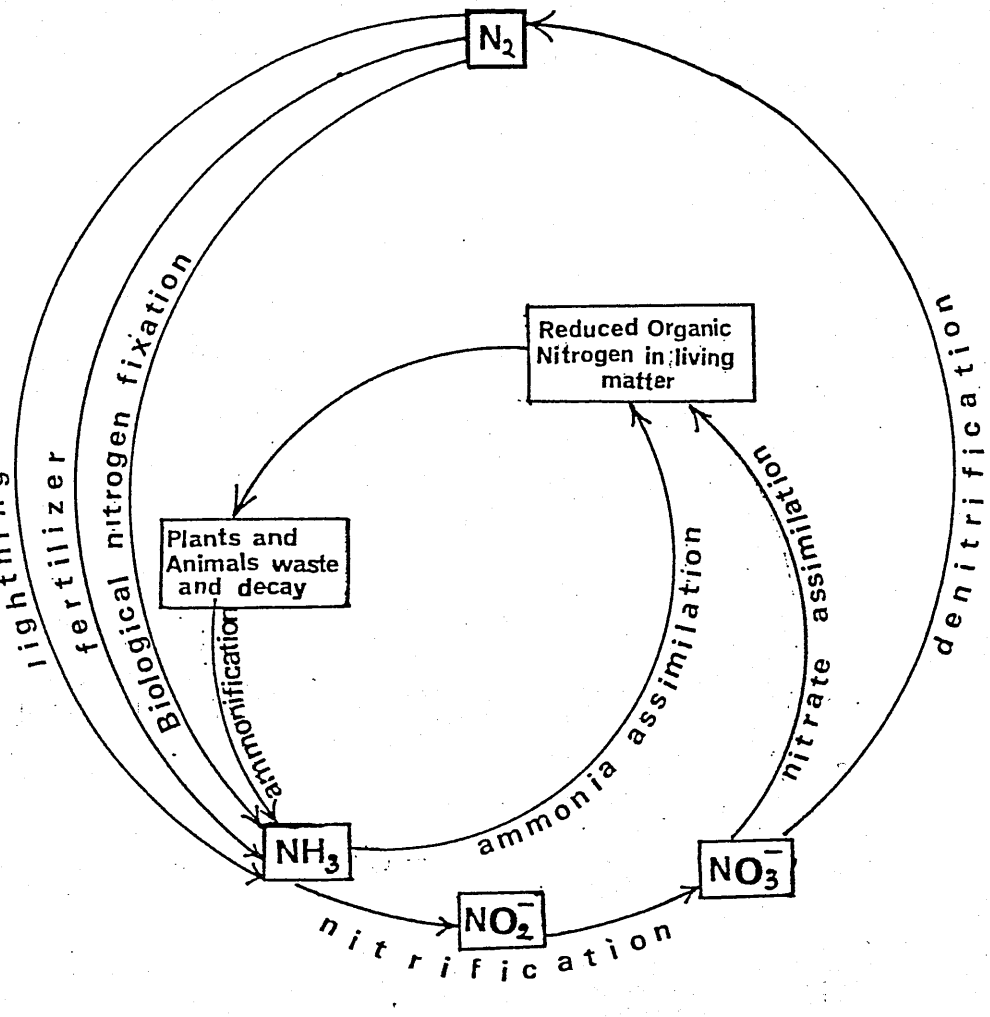


Fig 1.1. THE NITROGEN CYCLE (adopted from Brill, 1981)

Nitrification is brought about by 2 specialized groups of obligately aerobic chemoautotrophic bacteria; Nitrosomonas oxidizes NH_3 to NO_2^-; Nitrobacter oxidizes NO_2^- to NO_3^-.

Denitrification:- some bacteria can use NO_3^- instead of O_2 as a terminal electron acceptor. This process results in loss of combined nitrogen to the atmosphere.

Nitrate assimilation occurs in plants, many bacteria, some fungi and protozoa.

Ammonification involves the energy yielding oxidation of organic nitrogen compounds by various bacteria and fungi.

nutrition; soybean, French bean, peanut, pea, chickpea and cowpea are examples of such crops. Similarly the forage legumes such as clover and alfalfa contribute greatly to the nutritional quality of farm animals.

Rhizobium species which form nitrogen-fixing nodules in different legume hosts have been classified on the basis of their host specificity (Graham, 1964), although this is not always specific. The various Rhizobium species are listed in Table 1.2. Rhizobium species can also be divided into fast growers and slow growers by their growth rates on yeast extract medium (Jordan and Allen, 1974). The doubling time of the fast growers on conventional yeast agar extract medium at 30°C is 2-5 h whereas that for slow growers is 12-24 h. The fast growers form acid from mannitol, the slow growers do not. This grouping is not rigid, for example, Rhizobium lupini generally grows rapidly compared with other slow growers and on the other hand R. meliloti often grows sluggishly among fast growers.

Recently the root nodule bacteria have been classified into two genera, namely, Rhizobium and Bradyrhizobium (Jordan, 1984). Rhizobium species are considered fast growing with generation times of less than 6 h, whereas Bradyrhizobium strains have generation times in excess of 6 h (Jordan, 1982, 1984). The genus Rhizobium presently includes four species; R. fredii (Scholla and Elkan, 1984), R. meliloti, R. loti and R. leguminosarum. According to the new taxonomic classification R. leguminosarum includes the former species R. trifoli and R. phaseoli (Jordan, 1984).

Table 1.2. Species of root-nodule bacteria that form symbiotic relationship with various host legumes (Adopted from Jordan, 1984; Scholla and Elkan, 1984)

Host genus	Species of root-nodule bacteria		Growth on yeast mannitol medium
	Previous classification	Recent taxonomic classification	
Melilotus, *Medicago*, *Trigonella*	*Rhizobium meliloti*	*Rhizobium meliloti*	Fast growing (generation time about 6 h)
Lotus	*R. loti*	*R. loti*	
Pisum, *Vicia*, *Lens*, *Lathyrus*	*R. leguminosarum*	*R. leguminosarum*	
Phaseolus	*R. phaseoli*		
Trifolium	*R. trifolii*		
Glycine	*R. japonicum* (fast-growing)	*R. fredii*	
	R. japonicum (slow-growing)	*Bradyrhizobium japonicum*	Slow growing (generation time 12-24 h)
Lupinus	*R. lupini*		
Vigna, *Lotus*, *Arachis* and other tropical legumes	Cowpea miscellany		

R. fredii was earlier classified as "fast-growing" R. japonicum. Bradyrhizobium presently has one species, Bradyrhizobium japonicum and includes strains that are capable of effectively nodulating lupin and soybean. Also included within bradyrhizobia is the cowpea miscellany (Jordan, 1984) [see Table 1.2]. Although at present this is the standard accepted classification, this classification is not strictly followed in current literature. In discussions to follow, the "slow-growing" and the "fast-growing" soybean root nodule bacteria will be referred to as B. japonicum and R. fredii, respectively. The term "legume-Rhizobium" symbiosis will be used in a general sense to include all symbiotic associations involving legumes and root nodule bacteria under genera Rhizobium and Bradyrhizobium.

1.4. Structure and formation of a legume root nodule:

Rhizobia are found in most soils and are usually present in large numbers (10^3 to 10^6/g) in soils in which the appropriate host is grown. The process of infection and nodule formation has been discussed recently by several authors (Dazzo and Gardiol, 1984; Rolfe and Shine, 1984; and Downie et al, 1984). The location and topography of infection sites in soybean root hairs inoculated with B. japonicum have been studied recently in great details at the ultrastructural level by Turgeon and Bauer (1985). The plant and bacterium recognize each other by an unknown mechanism. Root hairs in various stages of development are the epidermal target cells

through which the bacterial infection begins. The bacteria attach along the sides of the root hairs and induce the root hair tip to curl. Bacteria entrapped within the tight curl penetrate the root hair cell wall and form a tubular structure called an infection thread. The infection thread passes through the outer cortical cells and then ramifies within the cortex. The infection thread passes through the cortex and grows towards the stele. At the same time proliferating cells which grow towards the infection thread become invaded by bacteria which are released from the tips of these threads and become enveloped in plant cell membranes. These infected cells then cease to divide and start to swell (Newcomb, 1980). An apical meristem is formed by the adjacent uninvaded cells which contiue to divide. This activity continues until a morphologically defined root nodule is formed. Pea nodules formed by R. leguminosarum and a longitudinal section of a pea nodule are shown in Figs 1.2 and 1.3 respectively. Within the infected cells the bacteria proliferate and differentiate into "bacteroids" (Figs 1.4 and 1.5), being enclosed inside an envelope, called the peribacteroid membrane.Simultaneously, the leghaemoglobin, an oxygen transporting protein (which will be discussed in chapter 2) is produced inside the nodule. Inside the bacteroid molecular nitrogen is reduced to ammonia by a chemical process which is catalyzed by the enzyme nitrogenase. The biochemistry of nitrogen fixation will be discussed in chapter 2. The ammonia is assimilated by the plant. For the process of

Fig 1.2 A photograph showing pea nodules formed by R. leguminosarum; magnification x3 (Courtesy N.J.Brewin). Pea nodules are indeterminate and continue to grow longitudinally as a result of apical meristem. In contrast the spherical nodules of many tropical legumes, eg soybean, cowpea, Phaseolus bean are determinate and expand radially as a result of peripheral cortical meristem.

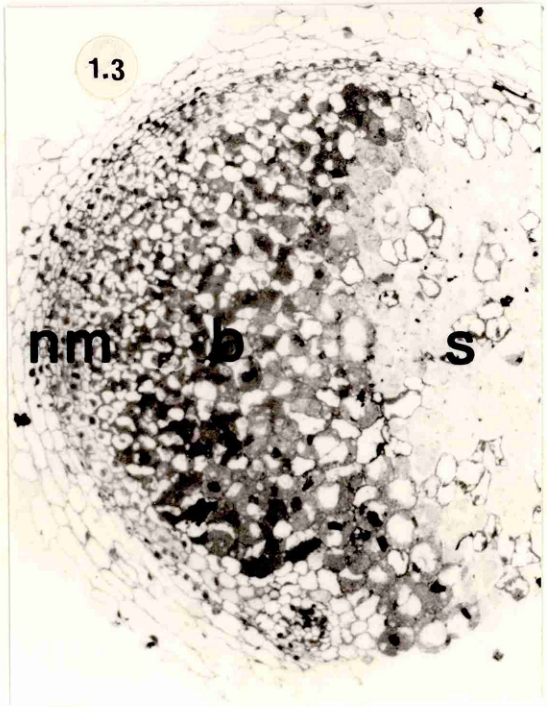
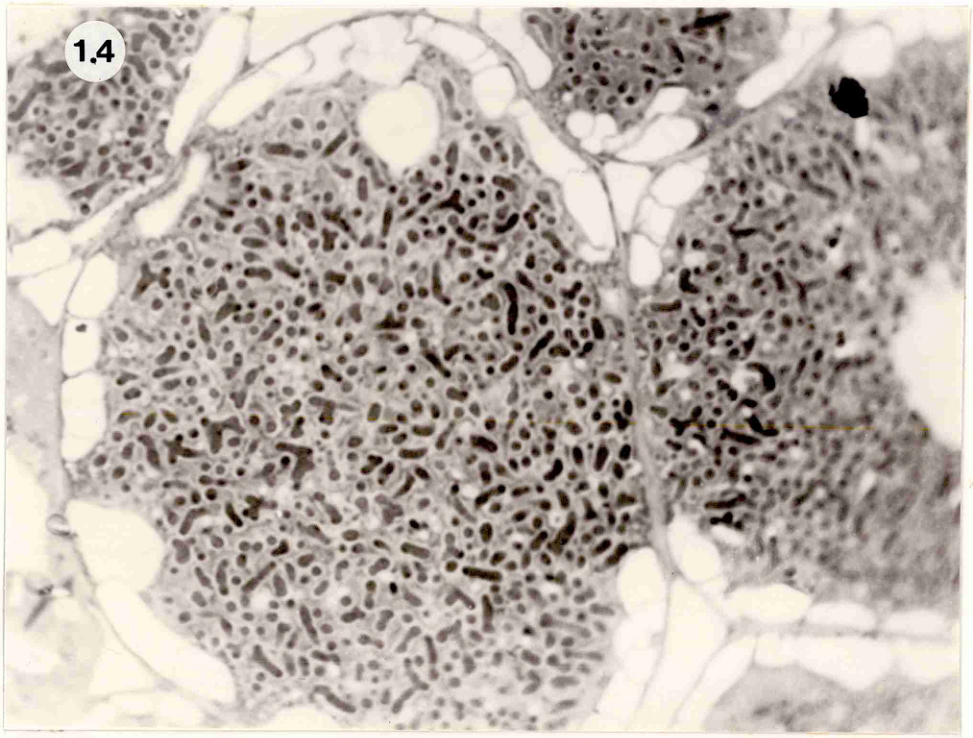


Fig 1.3 A longitudinal section of a pea nodule under simple microscope. The infected cells containing N_2 fixing bacteroids (b) are seen in the dark region between senescent cells (s) and nodule meristematic cells (nm); magnification x30 (Courtesy: N.J. Brewin)



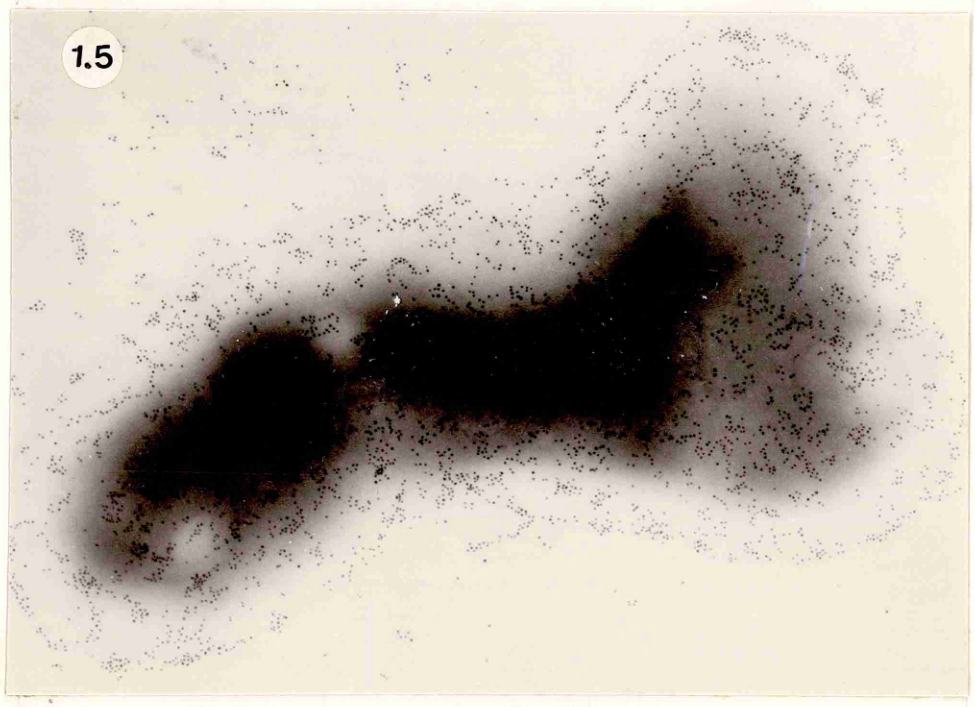


Fig 1.4. Photograph showing bacteroids inside infected host cells with peripheral starch grains; magnification x500. (Courtesy N.J. Brewin).

Fig 1.5 Electron micrograph of an isolated R. leguminosarum bacteroid from a pea nodule; magnification x 17,500. The lipopolysaccharide in the bacteroid outer membrane of the negatively stained bacteroid has been labelled with immuno-gold. (Courtesy N.J. Brewin).

nitrogen fixation bacteroids derive energy from the photosynthate supplied by the plant. The aspects of carbon metabolism in the bacteroids will be discussed in chapter 3.

1.5. Improving symbiotic N_2 fixation:

In order to improve existing symbiosis and to expand the range of potential hosts it would be necessary to understand symbiosis at the molecular level. In recent years considerable progress has been made in our understanding of the genetics and physiology of *Rhizobium*. Exploitation of symbiotic N_2 fixation through genetic manipulation will require a coordinated study of both genetics and physiology of the microsymbionts and the host legumes. The possibility of extending the N_2-fixing ability to cereals through the formation of a symbiotic relationship with N_2-fixing organisms may be a long term goal for future research.

There is a considerable variability among *Rhizobium* strains in their potential to fix N_2. In order to exploit this variability it is desirable to understand the genetic and physiologal basis of these variations. One such physiological trait in which there are great variations among the *Rhizobium* strains, is in their ability to recycle completely or partly the hydrogen evolved during nitrogen fixation. Some strains of *Rhizobium* possess an uptake hydrogenase system that can reoxidise the hydrogen evolved during nitrogenase reaction and generate ATP. Possession of an uptake hydrogenase may have some

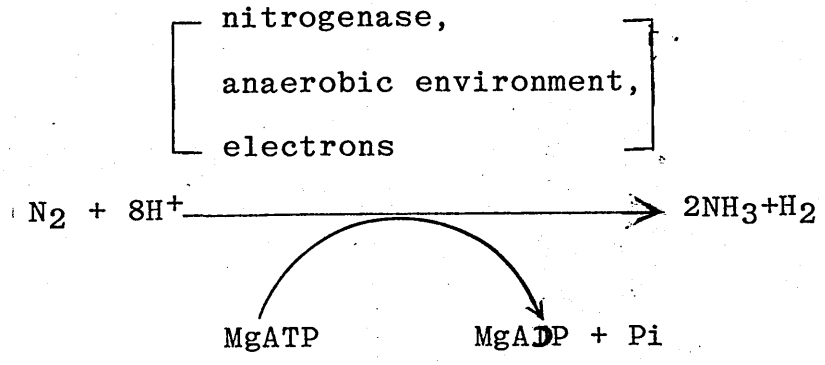
beneficial role in symbiotic nitrogen fixation. During the last few years a considerable number of investigations have been devoted to the understanding of the various aspects of uptake hydrogenase. It is important to review this literature in order to understand precisely the present status of knowledge about the possible beneficial role of uptake hydrogenase in symbiotic nitrogen fixation. This is the aim of this investigation.

Chapter 2

BIOCHEMISTRY OF NITROGEN FIXATION AND HYDROGEN EVOLUTION

2.1. Introduction:

The symbiotic nitrogen fixation due to legume Rhizobium association is a complex process carried out by the bacteroids inside the legume root nodules. In this process, reduction of atmospheric N_2 to NH_3 is catalyzed by the enzyme nitrogenase. The requirements for N_2 fixation can be shown simply by the reaction:



The main features of the biochemistry of nitrogen fixation are therefore :

i) the nitrogenase enzyme complex,

ii) an anaerobic environment for the oxygen-labile nitrogenase complex to function,

iii) electrons,

iv) the substrate: N_2 and H^+,

v) energy in the form of MgATP and

vi) in addition to ammonia, hydrogen gas is also produced.

The various biochemical processes associated with N_2 reduction are represented diagramatically in Fig 2.1. The current knowledge about these biochemical processes will be discussed in this chapter. The biochemical aspects of

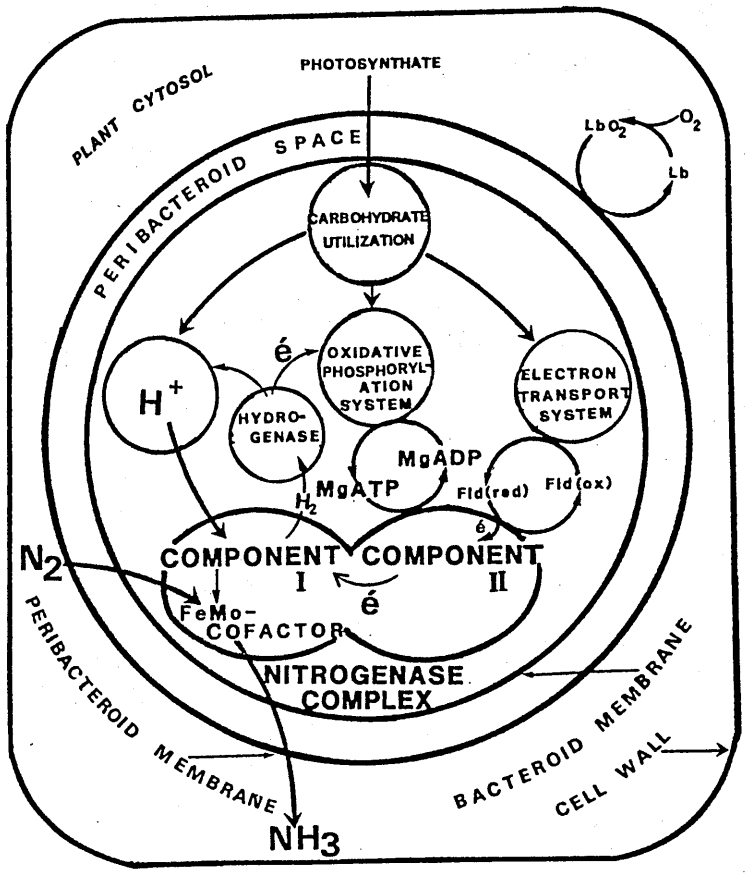


Fig 2.1. Schematic diagram of a Rhizobium bacteroid inside a nodule cell showing the inter-relationships of nitrogenase activity, carbohydrate utilization, electron transport and H_2 cycling. Carbohydrates from plant are the source of electrons, ATP and protons for N_2 reduction by the nitrogenase complex. Lb: leghaemoglobin, Fld(red): flavodoxin (reduced), Fld(ox): flavodoxin (oxidised).
[Based on the ideas of Brill (1977) and Robertson and Farnden (1980)]

nitrogen fixation have been considered in several reviews (Brill, 1980; Robson and Postgate, 1980; Yates, 1980; Eisbrenner and Evans, 1983).

2.2. Biochemistry of nitrogenase components:

The biochemical reaction in which N_2 is converted to NH_3 is catalyzed by the enzyme complex 'nitrogenase'. Nitrogenase is the characteristic enzyme common to all nitrogen fixing organisms. *Rhizobium* nitrogenase is found in the bacteroids of all *Rhizobium* species and also in the free-living strains of *B. japonicum* under microaerobic conditions. Two early developments made the biochemical studies of nitrogenase easier: (i) a method of protein extraction under anaerobic conditions, and (ii) the discovery of an assay system for nitrogenase using substrate analogue acetylene (C_2H_2) which is reduced by nitrogenase to ethylene (C_2H_4). A partially purified cell free extract of nitrogenase was first obtained from *Clostridium pasteurianum* (Carnahan *et al*, 1960) and was later found to be similar with the nitrogenase of other organisms. In all cases nitrogenase is made up of two soluble proteins which are called component I and component II (Bulen and LeComte, 1966; Mortenson *et al*, 1967; Vandercasteele and Burris, 1970; Eady *et al*, 1972; Shah and Brill, 1973).

2.2.1. Component I :

Component I is also known as MoFe protein or nitrogenase. It has a relative molecular mass 220,000 to 250,000 and contains 2 Mo atoms and 28 to 34 Fe atoms and

approximately 30 acid-labile sulphides (Eady and Postgate, 1974). Polyacrylamide gel electrophoresis of component I in sodium dodecyl sulfate has indicated that component I is composed of two subunits ($\alpha_2\beta_2$), each subunit having a relative molecular mass of approximately 60,000 (Nakos and Mortenson, 1971; Tso, 1974). Although from the amino acid analysis, the subunits appeared to be very similar to each other (Eady and Postgate, 1974), results from tryptic digestion patterns do not support common amino acid sequences of two subunits (Kennedy et al, 1976; Swisher et al, 1977; Lundell and Howard 1978). It seems possible that the two subunits could have resulted from a gene duplication and subsequent modification that may have occurred during evolution of nitrogenase (Brill, 1979, 1980). Each subunit of component I requires the other for stability in vivo. When a mutation in either gene makes one gene product unstable, the other gene product also is rapidly degraded (Roberts et al, 1978; Brill, 1980).

2.2.2. Iron Molybdenum Cofactor (FeMo-Co):

Component I contains an iron molybdenum cofactor which can be extracted with N-methylformamide (Shah and Brill, 1977). FeMo-Co can activate in vitro nitrogenase from certain Nif$^-$ mutants of Klebsiella pneumoniae (Nagatani et al, 1974; Roberts et al,1978; Shah et al, 1978,1984). FeMo-Co is believed to be the active site of nitrogenase. Purified component I has a unique paramagnetic resonance signal which is due to this FeMo-Co (Rawlings et al, 1978).

FeMo-CO contains a molybdenum iron cluster containing six Fe atoms to one Mo. Like FeMo-CO, the Mo-Fe cluster can reduce acetylene to ethylene in the presence of borohydride (Shah and Brill, 1981). This would indicate that a cluster of six Fe to one Mo is present in FeMo-Co, that is responsible for the active site of nitrogenase. In addition to the six Fe atoms in the FeMo cluster, the FeMo-Co has two extra Fe atoms, and thus Fe:Mo ratio in the FeMo-Co is 8:1 (Shah and Brill, 1977). All the molybdenum present in the nitrogenase component I is in the form of FeMo-Co (Shah and Brill, 1977). This together with the fact that no molybdenum containing intermediates in the biosynthesis of the cofactor have been identified, supported the idea that FeMo-Co is assembled on nitrogenase component I. However, Ugalde et al (1984) recently had shown that biosynthesis of FeMo-Co does not require the presence of nitrogenase component I. In its absence, FeMo-Co is accumulated on a different protein, presumably an intermediate in the normal FeMo-Co biosynthesis pathway.

FeMo-Co is extremely sensitive to oxygen. It is stable at room temperature as long as oxygen and water are absent. Although FeMo-Co is unstable in an aqueous environment, nitrogenase itself is stable. This suggests that component I protein core may be responsible for maintaining a protective environment at the active site of the FeMo-Co in component I (Shah and Brill, 1977). This cofactor has been isolated from component I of Azotobacter vinelandii, Clostridium pasterianum, Klebsiella

pneumoniae, Bacillus polymyxa and Rhodospirillum rubrum. In all cases FeMo-Co exibits identical properties (Shah et al, 1984).

2.2.3. Component II

Component II is known as Fe-protein or nitrogenase reductase (Hageman and Burris, 1978). The relative molecular mass of component II is 55,000 to 65,000 and is composed of two identical copies of single subunit (Eady and Postgate, 1974). Component II has two non-haem Fe atoms and four acid-labile sulfides. The amino acid sequence of component II from C. pasteurianum has been determined (Tanaka et al, 1977).

The two components join to form the active "nitrogenase enzyme complex". Component I from one organism can interact with component II from another to yield the active enzyme (Thorneley et al, 1975; Tsai and Mortenson,1978; Benson et al, 1979). Antiserum prepared against a component from a member of one genus can react with the component isolated from a bacterium of a different genus (Maier and Brill, 1976). Strong DNA homology among various organisms in the structural genes for nitrogenase (nifHDK) suggest common evolutionary origin of nitrogenase genes.

Substrate binding and reduction takes place on component I (Orme-Johnson et al, 1972; Smith et al, 1973). The role of component II is to supply electrons, one at a time to component I (Mortenson et al, 1973;

Ljones and Burris, 1978). No free intermediate of N_2 reduction have been found; however, there is some evidence that an enzyme bound dinitrogen hydride is an intermediate (Thorneley et al, 1978).

2.3. Electron supply to bacteroids:

The electron supply for nitrogenase comes ultimately from the carbon compounds supplied by the host. The nature of these compounds is gradually being elucidated. Mutants with particular lesions in catabolism have been used to associate metabolism of particular compounds with N_2 fixation. Dicarboxylate transport (dct) mutants are able to nodulate (Nod^+) but unable to fix N_2 (Fix^-) (Ronson et al, 1981) as are succinate dehydrogenase (sdh) mutants (Finan et al,1983), suggesting a specific role for succinate, fumarate or malate in fuelling N_2 fixation. However, details of pathways for the provision of the reductant and transfer of electrons to nitrogenase are not clear. Nitrogen fixation by nitrogenase occurs readily in vitro with a chemical reducing agent, hydrosulfite (Bulen et al, 1965; Dixon and Postgate, 1972). Electrons from carbon substrates drive nitrogenase activity in vivo. Ferredoxin and flavodoxin have been shown to donate electrons in vitro to nitrogenase (Benemann et al,1971; Yates, 1972; Yoch, 1974). In some cases there are two or more of these electron carriers in the cell (Yoch, 1974). Therefore it is difficult to ascertain which of these proteins actually donates electrons to nitrogenase in vivo.

2.4. Nitrogenase activity:

Nitrogenase has proved to be a remarkably complicated enzyme in both structure and mode of action; the latter is not wholly understood. The process of N_2 fixation involves the transfer of hydrogen atoms from carbohydrate such as glucose to the substrate N_2 at the site of nitrogenase enzyme complex (Fig 2.1). Theoretically in this process nitrogenase consumes six electrons and six protons to reduce one molecule of ammonia.

$$N_2 + 6H^+ \xrightarrow{6e^-} 2NH_3$$

However, there is evidence that electrons from nitrogenase can also be transferred to protons from the aqueous environment releasing molecules of hydrogen. Usually one molecule of hydrogen is produced during the reaction which would be more correctly represented as :

$$N_2 + 8H^+ \xrightarrow{8e^-} 2NH_3 + H_2$$

Electrons derived from carbohydrates are donated first to component II and then to component I where the actual reduction of N_2 to NH_3 takes place. For this process the nitrogenase enzyme complex requires energy which is in the form of ATP. 12 molecules of ATP are required for the reduction of one molecule of N_2 to NH_3. One molecule of H_2 is also produced and therefore an additional 4 molecules of ATP are required. Thus the energy requirement for reducing one molecule of N_2 to NH_3 is equivalent to 16 ATP molecules. A simplified scheme for this mechanism (Postgate, 1982) is discussed below and represented diagramatically in Fig 2.2.

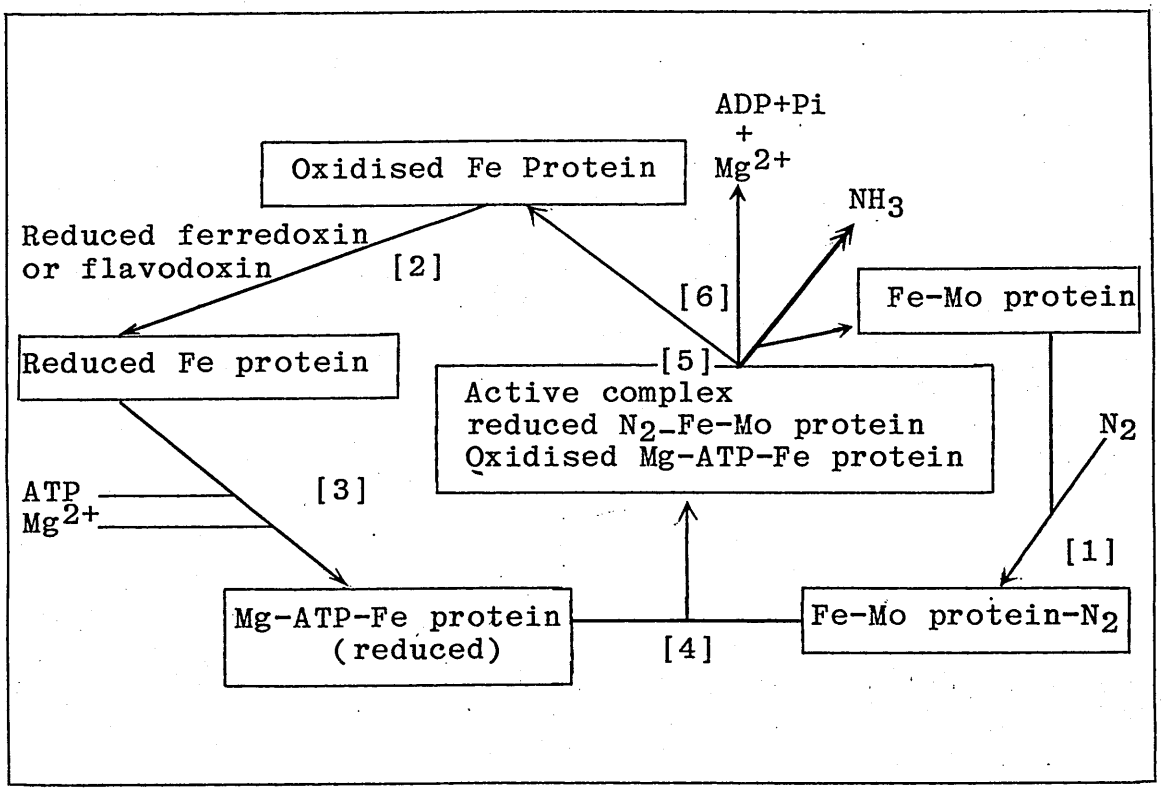


Fig 2.2. Schematic representation of events during turnover of nitrogenase.
[1] N_2 binds to Fe-Mo protein.
[2] Electrons transferred from ferredoxin or flavodoxin.
[3] Reduced Fe protein binds to MgATP.
[4] Complex forms between Fe-Mo protein and Fe protein.
[5] Electrons flow from Fe protein to Fe-Mo protein, coupled to ATP hydrolysis. N_2 reduced to NH_3
[6] The complex dissociates
(From Eady and Postgate, 1974)

The component II accepts an electron from a reduced ferredoxin or flavodoxin. The reduced Fe-protein (component II) reacts with the mono-magnesium salt as ATP and donates an electron to component I. The component I forms a complex with the Mg-ATP activated component II which is the "nitrogenase complex". Within the complex an electron transfers from the cluster of Fe-protein to the Fe atoms of its FeMo-Co of component I. The electron transfer process is probably accompanied by a loss of ADP, inorganic phosphate and Mg^{2+} as well as regeneration of component II. During this process the complex of the two protein components probably dissociates (Hageman and Burris, 1978). Chatt (1981) has recently proposed a simple model on the basis of a series of possible redox states for molybdenum which is bound to the active site of nitrogenase FeMo protein (Fig 2.3). In this model nitrogenase activity is based on a cycle of eight successive electron transfers from the Fe-protein to the active centre of FeMo protein (Thorneley and Lowe, 1981, 1982). Progressive reduction of the FeMo protein probably leads to a series of redox states for molybdenum (Chatt, 1981), of which only the tri-hydride state (MoH_3) is capable of binding substrate N_2 with the release of one molecule of H_2. The component I binds the substrate and holds it while electrons are added sequentially. In this way a total of eight electrons transfer into "component I-nitrogen complex" leading to sequential release of ammonia. In the absence of N_2, electrons combine with protons to yield H_2. Some of the other substrates that can be reduced by nitrogenase are listed in Table 1.

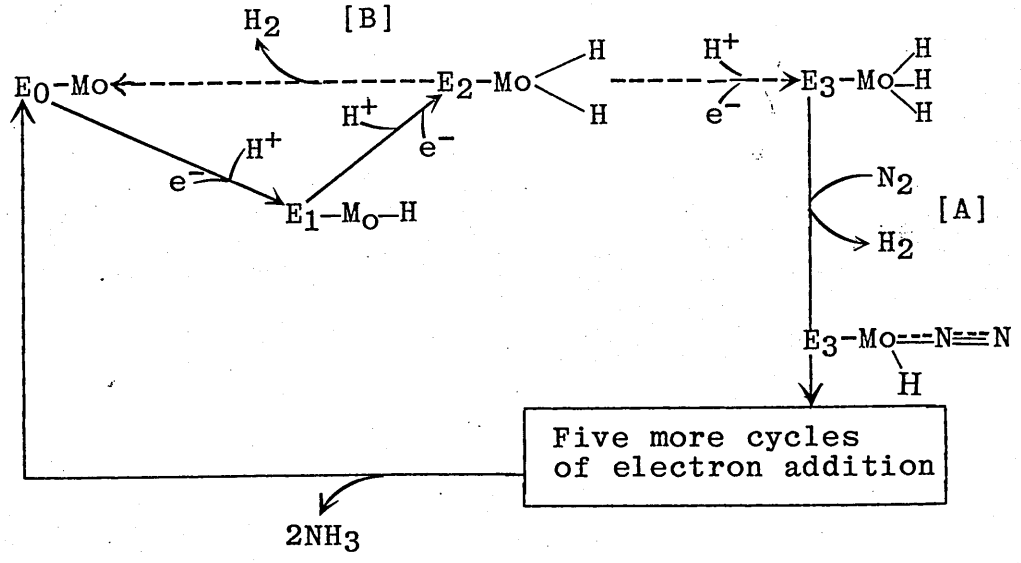


Fig 2.3. Model for nitrogenase action, illustrating the two main sources of H_2 evolution. [A] Stoichiometric evolution of 1 molecule of H_2 for each molecule of N_2 bound to the active site of nitrogenase FeMo protein. [B] Proton catalysed discharge of H_2, following dissociation of the Fe protein from the reduced FeMo protein. Eo-Mo represents component I in oxidised form. Component I with successive addition of one, two and three electrons from Fe-protein is represented respectively by E_1-Mo, E_2-Mo and E_3-Mo . Solid arrows, E_1—E_2 etc, represent a rapid electron transfer, followed in each case by a slower rate-limiting dissociation of oxidised Fe-protein from the reduced FeMo Protein. Progressive reduction of the FeMo protein probably leads to a series of redox states for Molybdenum (Chatt, 1981) of which only the trihydride state (MoH_3) is capable of binding sustrate N_2 with concominant release of one molecule of H_2. (From Brewin, 1984)

Table 2.1. Some substrates reduction catalysed by nitrogenase (Postgate, 1982)

Substrate	Products
N_2 (Dinitrogen)	$2NH_3 + H_2$
N_3 (Azide)	$N_2 + NH_3 + N_2H_4$
N_2O (Nitrous oxide)	$N_2 + H_2O$
CN (Cyanide)	$CH_4 + NH_3$
RCN (Alkyl cyanides)	$RCH_3 + NH_3$
RNC (Alkyl isocynides)	$RNH_2 + CH_4$
CH_2CHNC (Vinyl isocyanide)	CH_4
C_2N_2 (Cyanogen)	CH_4
C_2H_2 (Acetylene)	C_2H_4
RC_2H (Alkyl acetylene)	$RCHCH_2$
$CH_2C_2H_2$ (Allene)	CHC_2H_5
CH_2CHCN (Vinyl cyanide)	$CH_2C_2H_4 + NH_3$
$2H^+$ (Hydrogen ion)	H_2

2.5. The oxygen problem:

Bacteroids need oxygen for oxidative phosphorylation in the process of ATP generation which is an aerobic process, normally occurs in the presence of molecular O_2. This requirement poses one problem in legume-*Rhizobium* symbiosis, which is also common to other nitrogen fixing systems. Each of the two nitrogenase components is rapidly and irreversibly inactivated by exposure to O_2 (Bulen and LeComte 1966). The relationship between nitrogenase and oxygen is complicated by the observations that in certain bacteria, oxygen not only affects nitrogenase activity but also represses the synthesis of the enzyme (Eady *et al*, 1978; Robson,1979). Oxygen might also have indirect effects on nitrogenase, for example, by competing for reductant or by causing membrane peroxidation (Gallon, 1981). Oxygen damage causes alternations in various spectral characteristics of both nitrogenase components and it is likely that enzyme inactivation results from an interaction between O_2 and the reduced states of these proteins. Thus anaerobic conditions must be maintained during purification of the components.

Component II is more sensitive than component I. Oxygen inactivation renders the protein incapable of accepting electrons (Smith and Long, 1974). The FeMo-Co factor which can be extracted from component I is more oxygen labile than component II (Shah and Brill,1973). The nitrogenase proteins are not the only components of the N_2 fixing system which are oxygen sensitive, the electron carriers to nitrogenase, either ferredoxins or flavodoxins are also rapidly oxidized (Yates and Jones, 1974).

Atmospheric O_2 concentrations and active nitrogenase are incompatible, and therefore all nitrogen fixing organisms must protect their nitrogenase from the deleterious effect of O_2. In N_2-fixing root nodules respiration maintains a very low partial pressure of oxygen (0.1%) which is sufficiently low to avoid oxygen damage to nitrogenase. However, such a low oxygen concentration poses the problem of how to supply a sufficient flux of oxygen to the centre of the nodule tissue in order to sustain the high rates of respiration needed for N_2 fixation. This problem has been solved by the production of a red oxygen transport pigment leghaemoglobin.

2.5.1. Leghaemoglobin (Lb)

Inside the legume nodules the Rhizobium bacteroids are enclosed within the peribacteroid membrane (Fig 2.1). Surrounding this peribacteriod membrane, the plant cytoplasm contains large quantities of a pigmented molecule called leghaemoglobin. This is a monomer of relative molecular mass 15,000-17,000 which has a high affinity for oxygen and may constitute 10% or more of the protein in the plant cytosol fraction from legume nodules (Appleby, 1976; Begersen, 1977). For effective symbiotic N_2 fixation the nodules must contain leghaemoglobin. N_2-fixing nodules containing no leghaemoglobin have not been recorded, although mutant strains of Rhizobium with the ability to form leghaemoglobin containing nodules but unable to fix nitrogen, are known (Maier and Brill, 1976;

Bisseling et al, 1983). Leghaemoglobin is present only in the infected cells. Uninfected plant cells do not contain any trace of leghaemoglobin (Bisseling et al, 1983; Verma and Long, 1983). Therefore, the expression of leghaemoglobin gene appears to be under the control of rhizobia or nodule tissue formed after infection of plant by rhizobia. Thus leghaemoglobin is apparently a symbiotic product.

Several workers have reported that leghaemoglobin is located exclusively in the plant cytoplasm in nodules of various legumes (Robertson et al, 1978; Verma and Long, 1983; Bisseling et al, 1983), while some other studies have indicated that some leghaemoglobin is located in the peribacteroid spaces (ie the space between the bacteroid outer membrane and the peribacteroid membrane; see Fig 2.1) in some legumes (Begersen and Appleby, 1981, Begersen, 1982,1984). However, using immuno-gold staining of leghaemoglobin on thin sections of pea nodules Robertson et al (1984) have clearly demonstrated that leghaemoglobin can be detected in the plant cytoplasm and nucleus of infected cells but not in the peribacteroid spaces.

The globin protein of the leghaemoglobin are coded by plant gene and are synthesized on plant polysomes whereas the haem is produced by the bacteroids (Cutting and Schulman, 1971; Verma et al, 1979). The level of δ-aminolevolunic acid (δ-ALA) synthetase, the first enzyme of haem synthesis, increases in the bacteroids of soybean over the same period of time as the increase in leghaemoglobin in developing nodules, whereas its level

in the plant cytosol decreases during nodule development indicating that the bacteroid is the most likely site for haem synthesis (Nadler and Avissar, 1977). R. meliloti mutant strains with reduced levels of δ-ALA synthetase form small white nodules which do not fix nitrogen (Leong et al, 1982). Symbiotic effectiveness is restored in the mutant by supplementing the plant with exogenous δ-ALA or by introducing into the mutant the wild type δ-ALA synthetase gene cloned into a plasmid vector. The recombinant plasmid carrying the δ-ALA synthetase gene was also able to weakly complement an Escherichia coli haem-A mutant.

Leghaemoglobin has been purified from the root nodules of a number of legumes and it is always found to occur in multiple forms in all legumes. By separating the leghaemoglobin molecules of soybean nodules through isoelectric focussing, it was shown to consist of four major components (a, c_1, c_2, c_3) and four minor components (b, d_1, d_2, d_3) (Whittaker et al, 1981). The different leghaemoglobin may have slightly different functional properties (Bisseling et al, 1980). The different leghaemoglobin components may arise as the result of a number of gene duplications and several independent mutations within these duplicated genes (Brill, 1980). Studies of amino acid sequences of different leghaemoglobin components of lupin, soybean and animal globin revealed 8-13% differences within a species (Hyldig-Nielsen et al, 1982; Wiborg et al, 1982), 48% differeces between soybean Lba and lupin Lb1 and 85% differences between soybean Lba and animal globin

(Hunt et al, 1978). Despite these large differences, sequences alignment studies (Hunt et al, 1978) reveal that all leghaemoglobin and animal haemoglobin have conserved sequences in critical regions.

An oxygen carrying protein resembling Leghaemoglobin have recently been reported in nodules produced by Rhizobium on the non-legume Parasponia andersonii (Appleby et al, 1983) and by Actinomycete Frankia on other non-legumes (Tjepkema,1983).The leghaemoglobin like protein of Parasponia and soybean leghaemoglobin have no immunological cross reactivity but show close amino acid homology between many regions of the two proteins (Appleby, 1984). This similarity in amino acid sequences led the latter authors to conclude that the legume nodule leghaemoglobin and Parasponia leghaemoglobin lie close to each other in evolutionary history.

Leghaemoglobin is an oxygen binding protein which facilitates diffusion of O_2 in the bacteroid to support ATP synthesis (Appleby et al, 1975). In addition O_2 is transported as the Lb-oxygen complex, which means that at the bacteroid surface the equilibrium concentration of free O_2 is extremely low and unlikely to either inactivate nitrogenase or repress its synthesis. A rhizobial oxidase which results in efficient oxidative phosphorylation intercepts the oxygen.

The leghaemoglobin concentration is reduced in nodules with senescence. It is probable that many factors are responsible for the observed loss of leghaemoglobin

from such nodules; these factors include loss of photosynthetic substrate and consequent failure of the leghaemoglobin reductase mechanism, the appearance of nicotinic acid, the accumulation of nitrite or nitric acid which might cause haem degradation, and the appearance of active endopeptidases in senescing nodules (Puppo *et al*, 1980; Appleby *et al*, 1984).

2.6. H_2 evolution:

Nitrogenase can reduce a variety of small molecules in place of dinitrogen (Table 2.1). It can also reduce hydrogen ion (H^+) and thereby evolve H_2. Many N_2 fixing bacteria including *Rhizobium* evolve H_2 when they fix N_2. When electrons and ATP are available to nitrogenase but N_2 is absent, the electrons combine with protons to yield H_2 (Bulen *et al*, 1965). Burns and Hurdy (1975) compared nitrogenase to a biological battery powering the flow of electrons by hydrolysis of ATP. If N_2 happens to be around, it becomes reduced; otherwise the electrons reach the active site of the enzyme and merely recombine with protons. The substitution for N_2 with an inert gas such as argon over nitrogenase reactions markedly increases H_2 evolution, indicating that N_2 competes with protons for electrons at some point in the electron transport sequence or that the absence of N_2 makes available more sites for the reduction of protons. Both components of nitrogenase are required and ATP is hydrolysed during H_2 evolution (Mortenson, 1966). However, even when nitrogen is in the gas phase, some of the electrons are evolved as H_2, and

remaining electrons are used to reduce N_2 to NH_3 (Bulen et al, 1965). This is a property common to all nitrogenases that have been examined. The partitioning of electrons to N_2 reduction or H_2 evolution, in vitro, depends on the ratio of the component I and component II in the nitrogenase complex, the supply of electrons and the ATP concentrations (Silverstein and Bulen, 1970; Ljones and Burris, 1972; Hageman and Burris, 1978). Nif^- mutants of A. vinelandii are not able to evolve H_2 (Fisher and Brill, 1969). Thus H_2 evolution seems to share active site common to N_2 fixation.

In vivo upto about 50-60% electrons are lost as H_2 (Rivera-Ortiz and Burris,1975). Schubert and Evans (1976) have shown that most legume-Rhizobium associations waste about half of the electrons reaching the nitrogenase. The efficiency of nitrogen fixation could be improved if this hydrogen evolution could be eliminated.

The extent of H_2 evolution by nitrogen fixing organisms also varies widely. Hydrogen evolution during nitrogen fixation can be expressed as a function of total energy flux through nitrogenase. The term "electron allocation coefficient" (EAC) has been used to describe this relationship (Burns and Hardy, 1975).

$$EAC = \frac{\text{(Electrons to exogenous reducible substrate)}}{\text{(Total electron flux to exogenous substrate and protons)}}$$

When acetylene (C_2H_2) is used as the substrate, the EAC approaches 1.00, because H_2 evolution is virtually

undetectable. With N_2 as the substrate at physiological levels the EAC for nitrogenase is always less than 0.75. Another term, relative efficiency (RE) has also been used to reflect the extent of hydrogen losses during N_2 fixation (Schubert and Evans, 1976). Operationally RE is equivalent to the EAC (in the absence of other interacting enzyme systems).

$$RE = 1 - \left[\frac{\text{Rate of } H_2 \text{ evolution in air}}{\text{Rate of } C_2H_2 \text{ reduction}} \right]$$

In the absence of N_2 (as for example in a gas mixture in which N_2 is replaced by argon) all the electrons may be available for H_2 production, making the RE=0. In a fully efficient system in the presence of N_2, all electrons would be used for ammonia production; no H_2 would be evolved and thus RE would be 1.0. RE values ranging from 0.99 to 0.40 for different legumes were found. A few legumes (eg cowpea) had RE values approximately 1.0 . High RE could in most cases be associated with the particular Rhizobium strains used as inoculant (Carter et al, 1978). These apparently more efficient symbionts were subsequently found to possess an uptake hydrogenase system which recycled the H_2 formed and regenerated protons and ATP. The uptake hydrogenase system will be discussed in chapter 4.

It appears that H_2 evolution by nitrogenase is an inescapable by-product of nitrogen fixation. An explanation for the cause of H_2 evolution by nitrogenase comes from the suggestion that molybdenum of component I

can exist either mono-, di- or tri-hydride state (Chatt, 1981). Thus, the binding of every molecule of nitrogen to tri-hydride molybdenum releases one molecule of H_2 (Fig 2.3). Hageman and Burris (1980) suggested that H_2 evolution increases under condition of reduced electron flux through nitrogenase. When electron flow is low the molybdenum in active centre would be mostly in the dihydride or monohydride state. These forms are able to release H_2 after interacting with additional protons but they are unable to bind N_2.

The model of Chatt (1981) has been recently extended by Thorneley and Lowe (1981, 1982). It has been shown that after each electron transfer from the Fe-protein (component I) to the FeMo-protein (component II), the oxidised Fe-protein dissociates from FeMo-protein (Hageman and Burris, 1978). The revised model emphasises that this dissociation step is of critical importance to the overall level of H_2 evolution, because the oxidised Fe-protein protects the active centre of the FeMo-protein from reacting with protons and releasing H_2. However, dissociation of the Fe-protein following the second electron transfer brings the Mo at the active site into the dihydride state when it can react with protons releasing H_2 and thus prevents addition of the third electron.

Carbon monoxide (CO) has been shown to inhibit N_2 fixation, but not H_2 evolution (Rivera-Ortiz and Burris, 1975). Chatt (1981) proposed that CO also would be expected to displace two hydrogen atoms from the

enzyme-bound Mo-trihydride, forming a tightly bound MoCO complex which would block access to N_2. The third Mo-bound hydride ligand would remain free to react with a proton, forming H_2. This reaction would provide an explanation for the CO insensitivity of the catalysis of H_2 evolution by nitrogenase.

The process of H_2 evolution during N_2 fixation is energy expensive, since ATP and electrons are wasted. Besides, the H_2 evolved is an inhibitor of the nitrogenase reaction. The energetics of N_2 fixation and H_2 evolution will be discussed in the next chapter.

Chapter 3

CARBON SUPPLY TO BACTEROIDS

3.1. Introduction:

The reduction of N_2 catalyzed by nitrogenase in the root nodules depends upon the supply of host photosynthate to the nodules. The products of photosynthesis support the growth of nodules and provide ATP, reductant and the carbon skeletons for the removal of fixed nitrogen (Hardy and Havelka, 1975). The large flux of carbon through the nodule has been measured in nitrogen fixing pea plants during vegetative growth (Gordon et al, 1985). Several factors that decrease the amount of photosynthate available to the nodule have been demonstrated to decrease N_2 fixation, for example, decreased light intensity, partial defoliation, lodging and increased demands for support from other activities of the plant. Conversely, factors that increase the amount of photosynthate available to the nodule have been demonstrated to increase N_2 fixation (Hardy and Havelka, 1975). How the N_2 fixation in bacteroids is affected by the supply of photosynthate to the nodules and the aspect of photosynthate utilization in the bacteroids will be discussed in this chapter. These condiderations are very relevent to a discussion of the possible energetic advantages associated with the hydrogen recycling system which would be expected to reduce the amount of photosynthate required to provide the energy necessary to fix a given amount of N_2 (see chapter 6).

3.2. Experiments showing photosynthate as the limiting factor for N_2 fixation in legume root nodules:

Some experimental results demonstrating that nodule activity depends upon the supply of photosynthate are discussed below.

Evidence that photosynthate is a major limiting factor for N_2 fixation in field grown soybeans was obtained from a threefold CO_2 enrichment of the soybean canopy during the period of reproductive growth (Hardy and Havelka, 1975). The amount of N_2 fixed as determined by the acetylene reduction assay was increased from 75 to 425 Kg per hectare as a result of CO_2 enrichment. The major effect of CO_2 enrichment has been attributed to an increased net production of photosynthate made possible by the decrease in photorespiration brought about by the elevated CO_2 to O_2 ratio. Similarly, increased light intensity, a factor that increases the amount of photosynthate available to the nodule, has been demonstrated to increase N_2 fixation in the root nodules of pea (Bethlenfalvay and Philips, 1977, Sheikholeslam *et al*, 1980) and *Phaseolus* bean (Antoniw and Sprent, 1978).

Recently Gordon *et al* (1985) examined the flux of photosynthetic assimilate from leaves to nodules of soybean during N_2 fixation. A distinct pulse of ^{14}C-labelled assimilates was provided in this experiment through a supply of $^{14}CO_2$ to the first trifoliate leaf of soybean plants, followed by its excision at 60 min. A significant proportion of ^{14}C-labelled assimilate was exported to the nodules. The nodules accumulated ^{14}C at a

much greater rate than the shoots and roots, demonstrating that they were a strong sink for recent photoassimilates. Much less photosynthate was imported by nodules if nitrogen fixation was inhibited by low O_2 concentration. Thus the results of this experiment emphasize the close links between photosynthesis and nodule activity. Similar observations were made in another recent experiment by Kouchi et al (1985). In this experiment nodulated soybean plants were allowed to assimilate $^{13}CO_2$ for 10h in the light. The amount of $^{13}CO_2$ respired from nodules during the 10h $^{13}CO_2$ feeding period was 1.5 fold that of root respiration. These results demonstrated that the currently assimilated photosynthate was preferentially used to support nodule respiration, while root respiration relied considerably on earlier carbon reserved in the roots.

Ryle et al (1985a, 1985b) studied the short term response of nitrogenase activity to partial defoliation and photosynthesis in white clover. They observed that defoliation resulted in a fall in nodulated root respiration within 10 min; and more than 70% decline of nitrogenase activity within 1-2h. Nodule weight declined following defoliation and nodules on most severely defoliated plants exhibited accelerated senescence. The result of this experiment suggested that defoliation, a factor that decreases the amount of photosynthate available to the nodule, also decreases nitrogen fixation.

It is apparent from the experiments discussed above that N_2 fixation in the nodules may be adversely affected

by less than optimum supply of photosynthate to the nodule. Therefore in order to achieve a major increase in N_2 fixation, attention must be focussed on increased efficiency of photosynthate utilization. The efficiency of photosynthate utilization may be increased through processes such as CO_2 fixation and hydrogen recycling in the nodule. (H_2 recycling is a process in which the H_2 evolved as a byproduct of nitrogenase reaction is oxidized to regenerate energy in the form of ATP. The possible benefits of H_2 recycling will be discussed in chapter 6).

3.3. Photosynthate requirement in the nodules:

The nitrogen fixing root nodule is the major sink for the assimilates produced in the leaves of legumes. Photosynthate provides the energy and metabolites for growth and maintenance of nodules and bacterial cells, for nitrogenase activity and for ammonia assimilation. Theoretically, among various processes in the nodules, nitrogen fixation is the most energy intensive process consuming approximately 60% to 80% of total nodule ATP, while ammonia assimilation and transport are estimated to consume a further 20% (Pate et al, 1981). A proportion of carbon requirement for ammonia assimilation is met with the product of CO_2 fixation in the nodules.

3.3.1. Cost of N_2 fixation:

The synthesis of ammonia from molecular nitrogen and hydrogen is an exothermic reaction. Yet, this reaction requires a high energy input in both industrial as well as

biological systems. This is because the two atoms of the nitrogen molecule ($N \equiv N$) are linked by a triple bond, which is the strongest known covalent association between two atoms.

The biological reduction of N_2 catalyzed by the nitrogenase enzyme complex requires energy in the form of ATP in addition to a low redox potential reductant such as ferredoxin (Fd). The free energy released upon hydrolysis of ATP to ADP is essential for the reaction. The numbers of ATP molecules hydrolyzed for every electron-transfer to the substrate observed *in vitro* ranges from less than 2 to 24 (Ljones and Burris, 1972, Burns and Hardy, 1975, Salsac *et al*, 1984). The requirement of ATP changes with ATP concentration, the ratio of ATP to ADP, the ratio of Component II to Component I, temperature and relative efficiency (Ljones and Burris, 1972, Burns and Hardy, 1975, Davis *et al*, 1975). Considering a minimum of two molecules of ATP are hydrolyzed per e^- transfer and that electrons are supplied by reduced ferredoxin (Fd_{red}), the reaction will be:

$$N_2+16ATP+8Fd_{red}+10H^+ \longrightarrow 2NH_4^+ +H_2+16ADP+16pi+8Fd_{ox}$$

Assuming 1 mole of glucose is equivalent to 36 ATP, at least 0.44 moles of glucose is needed for the synthesis of 16 moles of ATP used per mole of N_2 reduced. Another 0.33 moles of glucose is needed for the reduction of Fd. On this basis the theoretical cost for 1 mole of N_2 reduction is equivalent to 0.77 moles of glucose (Schubert and Wolk, 1982).

However, the real cost of nitrogen reduction may

exceed the above estimate, depending on the relative efficiency (RE)[for definition see chapter 2]. At least one molecule of H_2 is produced per molecule of N_2 reduced. This minimum level of H_2 evolution corresponds to a RE value of 0.75. Schubert and Wolk (1982) estimated the theoretical cost of N_2 fixation for different values of RE considering one mole of glucose was equivalent to 36 ATP. They have shown that the cost of N_2 reduction is equivalent to 0.77, 0.98 and 1.17 moles of glucose at RE values of 0.75, 0.60 and 0.50 respectively. Based on the proportion of electron flow of nitrogenase to hydrogen evolution and the efficiency of uptake hydrogenase, Pate et al (1981) made theoretical estimates for the cost of nitrogen reduction (Table 3.1). These estimates ranged from 22.3 mol ATP/mol N_2 to 70 mol ATP/mol N_2 reduced. Expenditure increases steeply as electron allocation values rise above 40%, beyond which economics through hydrogenase become increasingly important.

Witty et al (1983) determined the respiratory cost of nitrogenase activity using the linear regression of respiration on nitrogenase activity in 13 legume species and recorded values from 2 to 5 moles CO_2/mole C_2H_2 reduced. They also observed variations in the carbon cost depending on Rhizobium strain and host cultivar.

Heytler and Hardy (1984) measured the metabolic cost associated with the symbiotic nitrogen fixation system in soybean nodules through a more direct method using a calorimeter constructed specially for this purpose by modifying standard laboratory equipment. By using this

Table 3.1. Estimated costs of N_2 fixation in legume nodules (cost as mol ATP/mol N_2 fixed) in relation to varying uptake hydrogenase activity and varying partitioning of electrons for proton and nitrogen reduction by nitrogenase (from pate et al, 1981)

		% electron flow of nitrogenase to H_2 evolution as opposed to N_2 fixation						
		10	20	30	40	50	60	70
% of evolved H_2 oxidized by uptake hydrogenase	0	23.3	26.3	30.0	35.0	42.0	52.5	70.0
	20	23.1	25.8	29.2	33.8	40.2	49.8	65.8
	40	22.9	25.4	28.5	32.6	38.4	47.1	61.6
	60	22.7	24.9	27.7	31.4	36.6	44.4	57.4
	80	22.5	24.5	27.0	30.2	34.8	41.2	53.2
	100	22.3	24.0	26.1	29.0	33.0	39.0	49.0

Note:- Operation of nitrogenase is costed as requiring inputs of the equivalent of 21 mol ATP/mol N_2 fixed and the equivalent of 7 mol ATP/mol of H_2 evolved; uptake hydrogenase is assumed to generate 3 mol ATP/mol H_2 oxidized.

method they estimated the cost of N_2 fixation to be 9.5 g of glucose per g of N_2 fixed, or 3.8g of carbon per g of N_2 fixed.

3.3.2.Cost of nodule growth and maintenance:

In both pea (Minchin and Pate, 1974) and cowpea (Rainbird et al, 1982), a strong correlation between e^- flow through nitrogenase and respiration has been shown. Observing a similar relationship between nodule respiration and nitrogenase activity in soybean, Rainbird et al (1984) experimentally determined the cost of total nodule function, as well as the individual cost of nitrogenase operation and nodule maintenance. In this study H_2 and CO_2 evolution from intact nodulated root systems under $Ar:O_2$ (80:20) atmosphere were measured under varied light regimes. The H_2 evolution approached zero after 36h of darkness at 22°C, while CO_2 evolution rate declined by 2.5 fold. Of the remaining CO_2 evolution, 62% was estimated to originate from nodules and represents a measure of nodule maintenance and respiration. The nodule maintenance requirement was temperature dependent and was estimated at 79 and 137 micromoles CO_2 per g dry weight of nodule per hour at 22°C and 30°C respectively. In this symbiosis nodule maintenance was estimated to consume 22% of the total respiratory energy. Recently Ryle et al (1984) have also measured respiratory costs of nitrogen fixation in nodules of soybean grown in a controlled environment and estimated the average respiratory cost to be 13.2 mg CO_2 mg^{-1} N. In this study, 20% of nodule respiration was

used for nodule growth and maintenace. The respiratory cost of N_2 fixation from these results may however be underestimated as CO_2 fixation was not accounted for in the measurement. In both studies (Rainbird et al, 1984, Ryle et al, 1984) the respiratory cost of nodule growth and maintenance was found to be much higher than theoretical estimates (Pate et al, 1981). A possible reason for this discrepancy between theoretical and experimental results may be that the nodule is operating partially anaerobically, thus requiring more CO_2 to be respired to produce an amount of energy similar to aerobic tissue (Rainbird et al, 1984).

3.3.3. Cost of nitrogen assimilation and transport:

Based on certain experimental observations Pate et al (1981) estimated the theoretical cost of NH_3 assimilation and transport to be approximately equivalent to 20% of the total ATP utilization in the nodules. However the estimation of the real cost of ammonia assimilation and transport is complicated by the fact that nodules of different legumes differ widely in the types and relative amount of nitrogenous solutes exported to the host plant, carbon compound translocated to the nodules, allocation of C to nodule dry matter and CO_2 fixation. In lupin-Bradyrhizobium association, asparagine constitutes 65.7% of the products exported from nodules while in cowpea-Bradyrhizobium association, ureide (37-49.2%) is the major compound exported from the nodule (Pate et al, 1981). It is also shown that cost of ammonia assimilation rises with

increasing involvement of ureides with the result that the net cost of NH_3 assimilation in cowpea-Bradyrhizobium is higher than in lupin-Bradyrhizobium association (Pate et al, 1981). However, there may be major saving in the transport cost of assimilate in the form of ureide having 4N atoms exported per ureide molecule than in amide with 2N per molecule (Layzell et al, 1979).

For assimilation of fixed nitrogen into asparagine and aspartate, the carbon skeletons are derived from oxaloacetate while for glutamate and glutamine the C is derived from α-ketoglutarate (Miflin and Lea, 1976). In lupin nodules it was estimated that one molecule of oxaloacetate would be converted to one molecule of asparagine for each N_2 molecule fixed (Scott et al, 1976). Oxaloacetate and α-ketoglutarate are tricarboxylic acid cycle (TCA) intermediates. However, any consumption of acids of the TCA cycle for ammonia assimilation would ultimately result in a shortage of oxaloacetate, and since oxaloacetate is the acetyl-CoA acceptor, this would lead to a build up of acetyl-CoA and the input of TCA cycle would stop (Walker, 1962). An alterative source of oxaloacetate is therefore needed. Christeller et al (1977) studied in vitro phosphoenolpyruvate carboxylase (PEPC) activity and in vivo CO_2 fixation in detached lupin root nodules and suggested that PEPC may be the major source of oxaloacetate for ammonia assimilation. Their results suggested that respiration was normally the major source of CO_2, fixed into oxaloacetate by PEPC.

3.3.4. Cost saving in nodule activity through CO_2 fixation:

In cowpea and alfalfa it has been demonstrated that nodules actively fix CO_2 through PEPC activity and thereby increase nodule carbon use efficiency by recycling a portion of CO_2 lost through nodule respiration (Layzell et al, 1979, Vance et al, 1983). Layzell et al (1979) determined the cost of symbiosis through a method involving simultaneous measurements of N_2 fixation, CO_2 and H_2 evolution by nodules of intact lupin and cowpea. The more efficient use of C by cowpea nodules, in their study, was due to a lesser requirement of C for synthesis of exported N compounds, a smaller allocation of C to nodule dry matter and lower evolution of CO_2. The activity of PEPC in nodule extracts and the rate of $^{14}CO_2$ fixation by detached nodules were several times greater for cowpea symbiosis resulting in higher degrees of CO_2 conservation than in lupin.

In alfalfa nodules CO_2 fixation is highly correlated with active N_2 fixation and has been shown to constitute up to 22% of carbon required for assimilation of symbiotically derived nitrogen (Vance et al, 1983). Both in vitro PEPC activity and in vivo CO_2 fixation activity in alfalfa nodules decrease following application of NO_3^- (Vance et al, 1983). PEPC has been purified from alfalfa nodules and characterized as a protein of relative molecular mass 97,000, constituting approximately 1-2% of the total soluble protein in actively N_2-fixing alfalfa nodules (Vance and Stade, 1984). Coker and Schubert (1981) investigated the extent and rate of CO_2 fixation in both

root and nodules of soybeans grown solely on symbiotically fixed N_2, a condition which favours the export of ureides and not asparagine. They observed that during the vegetative stages of growth of soybean grown symbiotically, CO_2 fixation in nodules increased at the onset of N_2 fixation but declined to a lower level prior to the decrease in N_2 fixation. This decrease coincided with a decrease in transport of amino acid, especially asparagine, and an increase in the export of ureides. Thus, these results support the observation that CO_2 fixation provides some carbon requirement in the nodules for ammonia assimilation.

From the above discussion it appears that many different approaches have been employed to assess the cost of N_2 fixation and assimilation experimentally. Therefore from these results it is difficult to accept any single estimate as the real cost of N_2 fixation and associated processes. Moreover, the total cost may vary in different legumes depending on the extent of CO_2 fixation and hydrogen recycling and whether asparagine or ureides are transported from nodules. An example of how the relative cost in lupins and cowpea vary as estimated by Pate et al (1981) is shown in Table 3.2.

3.4. Carbon metabolism in the bacteroids:

The ATP and electron supply for nitrogenase activity within the bacteroid comes ultimately from carbon compounds made available by the plant. The nature of these compounds and the pathway by which various carbohy-

Table 3.2. Respiratory flux associated with nodule functioning in two host:*Bradyrhizobium* associations (adapted from Pate *et al*, 1981).

Item of functioning	Proportion of respiratory flux associated with item mol/ATP,100g N_2 fixed	
	Lupinus albus: *Bradyrhizobium* WU425	*Vigna unguiculata*: *Bradyrhizobium* 176-A-27
"Growth" respiration	9	5
"maintenance" respiration	17	21
Transport of fixed N	18	17
NH_4^+ assimilation and related ^4C metabolism	19	20
Nitrogenase:Hydrogenase functioning	150-171	105-151

drates are metabolized in bacteroid are gradually being elucidated from studies of mutants with particular lesions of catabolism and ^{14}C-labelling experiments. Carbon metabolism in legume nodules has been recently reviewed by LaRue et al (1984) and Stowers (1985).

3.4.1. Sugar metabolism:

Legume nodules contain large quantities of sucrose and low concentrations of glucose and fructose (Streeter, 1980). The first growing rhizobia have an active disaccharide uptake system and inducible invertase and therefore sucrose can support their growth in free-living cells (Glenn and Dilworth, 1981). Bacteroids of R. leguminosarum from pea and of lupin-Bradyrhizobium from lupin are unable to utilize disaccharides (Glenn and Dilworth, 1981). However, by $^{14}CO_2$-labelling in Phaseolus vulgaris root nodules, Antoniw and Sprent (1978), demonstrated the presence of labelled sucrose, glucose and fructose in the bacteriods.

Reibach and Streeter (1983) studied the metabolism of translocated photosynthate in soybean nodules by $^{14}CO_2$-labelling. Plants were exposed to $^{14}CO_2$ for 30 minutes in the light followed by $^{12}CO_2$ for up to 5 hours. Two hours after the pulse, about 60% of the radioactivity in nodules was in a neutral fraction, and of this half was in the form of sucrose. The radioactivity in sucrose decreased with time and this was accompanied by an increase in radioactivity in α-trehalose. The radioactivity in sucrose in bacteroids declined more rapidly than did the radioactivity in sucrose in the cytosol fraction. These

results indicate that bacteroids of B. japonicum not only degrade sucrose, but also either accumulate it or reconstitute it rapidly from labelled precursors.

Lawrie and Wheeler (1975) studied the distribution of labelled metabolites in the nodules of V. faba after plant photosynthesis with $^{14}CO_2$ for 30 minutes. The neutral fraction contained 70% of the total radioactivity in nodule extracts and the label in glucose plus fructose accounted for 87.1% of the radioactivity in the neutral fraction. Bacteroids of R. leguminosarum isolated from pea nodules are incapable of transporting glucose (Hudman and Glenn, 1980, deVries et al, 1982) or oxidizing it (Glenn and Dilworth, 1981). Nevertheless they contain glucokinase, and Entner-Doudoroff pathway enzymes 6-phosphogluconate dehydratase and 2-keto-3 deoxy-6 phosphogluconate aldolase (Glenn et al, 1984b). The specific activity of those enzymes in bacteroids was about half of that found in free living bacteria grown on glucose. The bacteroids contained 6-phosphogluconate dehydrogenase, and hence may have a pentose phosphate pathway. The bacteroids, like glucose grown cells, lacked phosphofructokinase and thus do not have the Embden-Meyerhof pathway.

Tn5 transposon mutants lacking glucokinase, fructokinase and pyruvate dehydrogenase have been isolated from R. leguminosarum (Glenn et al, 1984a). Although these mutants are unable to utilize sugars, they all nodulate peas and fix N_2. This strongly suggests that the capacity to utilize particular C_6 and C_{12} sugars is not essential either to establish the symbiosis or to support fixation.

Ronson and Primrose (1979) found that glucose- or fructose-negative mutants of R. trifolii were able to nodulate and fix N_2. However, Duncan (1981) reported that a fructokinase mutant of R. meliloti nodulated but was unable to fix N_2.

3.4.2. Organic acid metabolism:

Organic acids are considered the primary energy source for bacteroids (Ronson and Primrose, 1979; Ronson et al 1981; Gardiol et al, 1982). The presence of the TCA cycle in bacteroids has long been established (Stovall and Cole, 1978). That dicarboxylic acids are necessary for complete functioning of bacteroids is indicated by experiments using mutants of three Rhizobium species. A mutant of R. meliloti unable to grow on L-arabinose, acetate or pyruvate and lacking a α-ketoglutarate dehydrogenase, forms ineffective nodules of alfalfa (Duncan and Fraenkel, 1979). Mutants of R. trifolii defective in C_4-dicarboxylate transport are unable to grow on or transport succinate, fumarate or malate in the free-living stage and formed white ineffective nodules on clover plants (Ronson et al, 1981). Similarly C_4-dicarboxylic acid transport mutants of R. leguminosarum formed nodules on pea with no or reduced nitrogenase activity (Finan et al, 1983). A succinate dehydrogenase defficient mutant is also isolated from R. leguminosarum that formed ineffective nodules on pea (Finan et al, 1983). Similarly, a succinate dehydrogenase mutant of R. meliloti showed delayed nodulation and formed white ineffective nodules on alfalfa (Garidol et al, 1982).

These results strongly suggest that dicarboxylic acids play the central role in bacteroid metabolism. The metabolites such as succinate, formate or malate might play specific roles in fuelling N_2 fixation. This would indicate that a complete TCA cycle is required for effective nitrogen fixation. The results do not however resolve the problem of what carbon compounds the rhizobia obtain during the infection process. The fact that the mutants are capable of infection, division and pleomorphic change indicates that they are capable of obtaining adequate carbon compounds for growth.

Other organic acids such as formic acid and malonic acid can also serve as a carbon source for growth in B. japonicum and can be oxidized by bacteroids isolated from soybean (Manian et al, 1982). A mutant strain of B. japonicum unable to grow on formate was found to lack formate dehydrogenase (Manian et al, 1982). The mutant did not reduce acetylene ex planta when formate was a carbon source. The strain formed effective nodules, indicating that formate does not play an essential role in the bacteroid metabolism. Similarly malonate is not an important metabolite and is probably not oxidized by bacteroids under normal physiological conditions. This conclusion is drawn from the results of $^{14}CO_2$ labelling experiments, by Reibach and Streeter (1983) who found only traces of label in malonate after $^{14}CO_2$ feeding to the soybean shoot.

Rhizobium bacteroids are able to store and metabolize carbon as poly-β-hydroxybutyrate (PHB) (Wong and

Evans, 1971; Karr et al, 1984). PHB is depolymerized to β-hydroxybutyrate which is dehydrogenated to form acetoacetate. Acetoacetate is ultimately oxidized via the TCA cycle (Wong and Evans, 1971). Karr et al (1984) have recently investigated the metabolism of PHB and the operation of glyoxylate cycle under nitrogen-fixing conditions. They found that the increase in the N_2 fixation activity was correlated with an increase in PHB content, β-ketothiolase, fumarase and β-hydroxybutyrate dehydrogenase and a decrease in isocitrate dehydrogenase and acetoacetate-succinyl-CoA transferase activities. The nature of the interactions of these enzymes and nitrogen fixation is unclear at this time.

The metabolism of aldehydes and alcohols in Rhizobium bacteroids has been investigated. Aldehydes and alcohols are present in soybean nodules and can support nitrogen fixation in isolated bacteroids (Peterson and LaRue, 1981, 1982). Aldehyde dehydrogenase activity has been detetected in B. japonicum bacteroids (Peterson and LaRue, 1982).

It appears that in both free living and symbiotic states rhizobia are capable of utilizing a variety of sugars and organic acids and therefore are likely to be confronted with a mixture of potential carbon sources. It is however not known whether bacteroids in the nodule possess any mechanism for catabolite control or selection of substrate although there are reports on control of substrate selection by free living Rhizobium (Dilworth et al, 1983).

Chapter 4

OTHER FACTORS LIMITING N_2 FIXATION IN LEGUME ROOT NODULES

In order to evaluate the significance of hydrogen recycling to the overall efficiency of N_2 fixation in legume root nodules, it is first necessary to consider what factors normally limit nodule activity and the conditions under which they operate. In addition to the supply of photosynthate to the bacteroids within the root nodules, there are several other environmental and genetic factors which can influence nodule formation and N_2 fixation by Rhizobium. These will be discussed in this chapter.

4.1 Environmental factors:

Several environmental factors can affect nodulation and N_2 fixation by Rhizobium, for example, fixed nitrogen in the soil, other mineral nutrients, soil temperature and moisture. N_2 fixation in the nodule is also influenced by oxygen concentration and hydrogen evolution inside the nodule.

4.1.1 Fixed nitrogen in the soil:

Legumes form nodules and fix N_2 when there is insufficient fixed nitrogen in the soil to supply the growth requirements of the plant. When adequate mineral nitrogen is available in the soil, nodule formation and the N_2 fixation activities are slowed down or completely stopped.

4.1.1.1 Effect of fixed nitrogen on nodulation:

Excess of NH_4^+ or NO_3^- completely prevents nodulation. In Vicia faba there was a small but consistent drop in nodule fresh weight of plants grown in sand culture and fed with nutrient solution containing 6 mM NO_3^- (Hudd et al, 1980). Application of 150 kg N/ha was found to be sufficient for complete inhibition of nodulation in Phaseolus vulgaris, at least until flowering (Taylor et al, 1981). Roughley et al,(1983) observed that in V. faba grown in sand culture, about 250 ppm N as NO_3^- flushed through twice daily was necessary to inhibit nodulation completely. Harper and Gibson (1984) studied the effect of different levels of external NO_3^- on nodulation of soybean grown on nutrient solution containing NO_3^- levels varying from 0.5 to 40 mM in different treatments. They observed that even at 1.0 mM nitrate concentration (resupplied daily to initial level) the appearance of nodules was retarded. Higher external nitrate levels were inhibitory to nodule appearance even though nitrate uptake rates among different treatments were similar.

Recently, Gibson and Harper (1985) studied the effect of nitrate on nodule appearance in soybean inoculated with strains of B. japonicum. Soybean plants were grown hydroponically in controlled environment cabinets. There were six treatments involving different levels of $NaNO_3$ ranging from 0 to 4.0 mM concentrations. NO_3^- was either supplied daily ("maintained") during the growth period or allowed to

deplete with plant growth ("rundown") ; the delay was more severe at higher nitrate concentrations. The external concentration of nitrate rather than the rate of nitrate uptake appeared to have a major effect on the initial stages of nodulation.

One way that N_2 fixation in legumes is regulated by fixed nitrogen is at the level of nodulation (Bishop et al 1975; Brill, 1980; Harper and Gibson,1984). However, how fixed nitrogen in the soil prevents nodulation is not fully understood. It was suggested that nitrate retarded nodule development through inhibition of root hair infection (Munns, 1968). Indole-3 acetic acid (IAA) produced by Rhizobium from tryptophan in the root exudates, is probably involved in root hair infection and nodule initiation (Kefford et al, 1960). This and the observation that most rhizobia can reduce nitrate to nitrite, prompted Tanner and Anderson (1963,1964) to propose that the inhibitory action of nitrate was based upon the catalytic destruction of IAA by nitrate. Support for this hypothesis was provided by studies in which supplemented IAA in the culture medium alleviated the inhibitory effects of nitrate on nodulation (Valera and Alexander,1965; Munns, 1968). Another suggestion as to how fixed N inhibits nodulation is based on the idea of possible involvement of lectin, which is produced by root hairs, in Rhizobium-host recognition (Dazzo and Brill, 1978). It was proposed that fixed N possibly prevents the lectin from being available to the bacteria. An alternative explanation

for the inhibition of nodule growth by nitrate came from the experimental results of Streeter (1985a,1985b). He studied the effects of various concentrations of nitrate on the N content of nodules as ammonium, amino acid, ureide and also on the N transport rate from roots to shoots. His results showed that a high nitrate supply (15mM concentration) in the nutrient solution resulted in depression of nodule growth and that this was accompanied by an accumulation of high concentrations of nitrate and low concentrations nitrite in soybean nodule. These results suggest that the inhibition of soybean nodule growth by nitrate may be directly associated with nitrate metabolism, possibly with accumulation of some amount of nitrite. The question of how high concentrations of combined nitrogen affect nitrogen metabolism will be addressed in section 4.1.1.2.

From the above discussions, it appears that a high concentration of combined nitrogen retards or completely inhibits nodule formation. The exception to this is that small doses of "starter N" may promote nodulation during early seedling growth, because the development of these seedlings may be N-limited in the absence of a functional system for symbiotic N_2 fixation. A small dose of N is considered useful for overcoming the transient nitrogen stress of germinating seedlings during the first two weeks when the symbiotic N_2 fixation system is not operational. Koermendy and Eaglesham (1984) studied the effect of different doses of starter N on nodulation of soybean by seven R. japonicum strains. They observed that the effect of N depended on the amount

of starter N applied, the Rhizobium strains and the plant age. In this study, a dose of 90 mg KNO_3 per pot at 14 days after planting caused a reduction of nodule number with four out of seven strains, whereas at 24 days after planting the same dose of nitrate increased nodule number by more than 100% with six of the strains. Thus, a low level of combined nitrogen is not detrimental to nodule formation but may be even beneficial for initial nodule formation. Apparently, legumes form nodules and fix N_2 only when there is a great demand for fixed N in the plant in the absence of the required amount of inorganic nitrogen in the soil. The process of nodule formation and N_2 fixation is probably more expensive for the plant than the uptake and incorporation of nitrate.

4.1.1.2 Effect of fixed nitrogen on N_2 fixation :

In K. pneumoniae and other free-living nitrogen-fixing organisms, it has been established that exogenous ammonia and nitrate or nitrite rapidly "switch off" the nitrogenase activity (Postgate, 1982). Nitrate exerts repressive effects on nitrogenase synthesis in K. pneumoniae by acting as a source of ammonia (Drozd et al, 1972) or via the oxygen pathway (Hom et al, 1980). In various legume-Rhizobium symbioses it has been well established that a high concentration of combined inorganic nitrogen in the soil suppresses the nitrogenase activity. The biological regulation of N_2 fixation in the nodule by the effect of fixed nitrogen may not be exactly similar to that in K. pneumoniae or other free-living organisms. Although there has been some

interest in the effect of NH_4^+ in the legume-*Rhizobium* symbiosis, most of the emphasis has been focussed on the inhibitory effect of NO_3^- upon nodule activity. From the available literature the possible ways in which high levels of fixed nitrogen represses nitrogen fixation in the bacteroids can be summarised as follows:

(i) Nitrate may exert a direct deleterious effect on nitrogenase by producing nitrite, which can irreversably damage the nitrogenase (Kennedy *et al*, 1975).

(ii) Wilson (1940) proposed that the internal carbohydrate to nitrogen (C/N) ratio governs nodule formation and N_2 fixation. The low C/N ratio in the presence of nitrate reduces both nodule formation and nitrogen fixation.

(iii) Nitrogen-fixing nodules and nitrate assimilating centres (nitrate reductase) may compete for common cofactors such as ATP, reductant and C-skeletons, resulting in reduced supply of these factors to the bacteroids (Small and Leonard, 1969; Oghoghorie and Pate, 1971).

(iv) Combined nitrogen induces root nodule senescence in terms of nitrogenase activity and leghaemoglobin content (Chen and Phillips, 1977)

(v) High combined nitrogen interferes with ammonia assimilation from the root nodule by reducing the nodule enzymes - glutamine synthase and glutamate dehydrogenase (Becana *et al*,1984). This may lead to nodule senescence.

The mechanism by which nodule growth and nitrogenase activity are affected by nitrate is not very clear. One of the simplest explanations for the effect of nitrate on nodule growth and nitrogenase activity suggests that this is due to nitrite, an intermediate product of nitrate metabolism. Nitrite is believed to cause inhibition of N_2 fixation, because: (a) It is a potent inhibitor of nitrogenase activity when mixed with the purified enzyme (Trinchant and Rigaud, 1980) or with bacteroids *in vitro* (Kennedy *et al*, 1975; Rigaud and Puppo, 1977; Trinchant and Rigaud, 1984) or when supplied to detached nodules (Stephens and Neyra, 1983); (b) It oxidizes leghaemoglobin *in vitro* resulting in the formation of ferric leghaemoglobin (Rigaud and Puppo, 1977); and it (nitrous acid) reacts spontaneously with primary and secondary amines (Sreeter, 1982).

The nitrite accumulation in cowpea nodules suppl-ed with high (15 mM) nitrate concentration in nodules has been shown to be positively correlated with nitrate reductase activity in bacteroids (Manhart and Wong, 1980). Therefore, nitrate reductase could be responsible for the observed inhibition of N_2 fixation when nodulated plants were exposed to nitrate. However, Gibson and Pagan (1977) showed that growth and N_2 fixing activity of nodules formed by nitrate reductase deficient mutant strains of cowpea-*Bradyrhizobium* and *R. trifolii* were still depressed when plants were supplied with nitrate. Stephens and Neyra (1983) also reported similar results with nitrate reductase deficient mutants of *B.japonicum*. In both these studies (Gibson and Pagan,1977; Manhart

and Wong, 1980) nitrite was not detected in the nodules formed by rhizobia lacking nitrate reductase. These observations make it less likely that the generation of nitrite in nodules may be the mechanism by which nitrate inhibits nodule growth and activity. Also bacteroids of many Rhizobium species apparently lack nitrate reductase (Manhart and Wong, 1979). This would suggest that bacteroid nitrate reductase need not be involved in the depression of nodule growth and nitrogen fixation. However, the possibility that nitrate reductase which is of plant origin may be involved in producing nitrite within root nodules can not be ruled out from any of these experiments. Streeter (1982)observed a positive correlation between nitrate reductase (NR) activity in the nodule cytosol and the amount of nitrate applied to the soybean plants inoculated with either NR deficient or NR positive strains of Rhizobium. Also nodules of plants not supplied with nitrate also contained significant amount of cytosol NR (Streeter,1982).

Recently Becana et al (1985a) have observed that R. meliloti bacteroids from alfalfa root nodules contained high specific activities of NR and nitrite reductase (NiR). Both enzymes were presumably substrate induced, although substantial enzyme activities were present in the absence of NO_3^- .In the presence of NO_3^-,the specific activity of NR was considerably greater than that of NiR, thus causing NO_2^- accumulation in bacteroids. Accumulation of NO_2^- within bacteroids indicates that NO_2^- inhibits nodule activity after feeding plants with NO_3^-. In another recent report

Becana et al (1985b) have shown that in nodules of alfalfa, proteins from plant cytosol and bacteroid were reduced by 30% upon exposure of the nodules to NO_3^- concentrations above 20mM. They observed that both NO_2^- and NO_3^- concentratios in the bacteroid fractions of alfalfa nodules increased with increases in the NO_3^- concentrations supplied to the nodules. They suggest that in the nodule, the capacity of NO_2^- reduction is not sufficient to avoid accumulation of NO_2^- and support the view that NO_2^- plays a more important role than NH_4^+ or NO_3^- itself as inhibitor of nodule activity.

The C/N ratio hypothesis (Wilson, 1940) and the photosynthate deprivation hypothesis (Small and Leonard, 1969; Oghoghorie and Pate, 1971) have predicted the inhibitory effects of nitrate on N_2 fixation. If these hypotheses were correct, adding carbohydrate to the growth medium should alleviate the inhibitory effect of nitrate. Rigaud et al (1973) reported that succinate at about 25 mM concentration stimulated both NO_3^- and C_2H_2 reduction in the bacteroid of B. japonicum under anaerobic conditions. They observed that glucose concentrations up to 120 mM stimulated C_2H_2 reduction but NO_3^- reduction was inhibited in the presence of glucose. Wong (1980) studied the effect of fructose, glucose and sucrose added to the growth medium on nodule number, nodule size and acetylene reduction activity of lentils grown in N-free or NO_3^- solution. The result of this study showed that sugar alleviated some inhibitory effects of nitrate. Thus these findings (Rigaud et al, 1973; Wong, 1980) supported the "C/N ratio" and

"photosynthate deprivation" hypotheses mentioned above. However, Chen and Phillips (1977) reported that increasing photosynthate supply in pea plants by carbon dioxide enrichment can not alleviate the inhibitory effects of nitrate. They concluded that NO_3^- does not inhibit symbiotic N_2 fixation through competition between nitrate reductase and nitrogenase for photosynthate. On the other hand, they observed that root systems supplied with 100mM KNO_3 or NH_4Cl exhibited a decrease in nitrogenase activity followed by a decline in the leghaemoglobin content. The reduction in the nitrogenase activity in this study may be interpreted as the consequence of deleterious effect of nitrite on leghaemoglobin. It has been demonstrated from the studies of Rigaud and Puppo (1977) that high nitrite levels could depress N_2 fixation by convertion of leghaemoglobin into an inactive form. Thus the oxidation of leghaemoglobin into an inactive form or direct inactivation of nitrogenase by nitrite, appears to be the most reasonable explanation for the effect of combined nitrogen on N_2 fixation. The suggestion that high combined nitrogen interferes with the enzymes for ammonia assimilation (Becana et al, 1984) requires further research.

4.1.2. Other mineral nutrients :

Nitrogen fixation in legumes is affected by a range of mineral nutrients. The effects of some of the mineral nutrients are briefly discussed below:

(i) Phosphorus:- It has been suggested that phosphorus (P) deficiency limits N_2 fixation mainly by reducing the growth of host plants (Munns, 1977; Graham and Rosas, 1979). This view is supported by observations of the early responses to P addition on shoot growth, nodulation and N_2 fixation in subterranean clover (Robson et al,1981). Recently, Jakobsen (1985) studied the influence of P on N_2 fixation and dry matter production in pea. Nodule dry weight, C_2H_2 reduction and P concentration in shoots responded to P addition before any growth response could be observed. A supply of P to P-deficient plants increased both the nodule dry weight and C_2H_2 reduction. The reduced nodulation and N_2 fixation in P-deficient plants seemed to be caused by impaired shoot metabolism and not by a direct effect of P deficiency in the nodules.

(ii) Molybdenum:- Since molybdenum is a component of nitrogenase, its presence is clearly necessary for activity. In K. pneumoniae, Brill et al (1974) observed that molybdenum deficiency led to the repression of nitrogenase synthesis. It is also well established that the requirement for molybdenum in the root nodules of legumes exceeds requirements elsewhere in the plant (Robson, 1983).

(iii) Cobalt :- Nitrogen fixing activity of cobalt deficient root nodules is limited primarily by inhibition of bacteroid multiplications (Dilworth et al,1979). Riley and Dilworth (1985a,1985b) have investigated the cobalt requirement for nodule development and observed that the nodule development was limited by severe cobalt

deficiency. Leghaemoglobin synthesis appeared to be sensitive to cobalt limitation. They estimated the critical root cobalt level for the normal symbiosis to be 300-500 g Co g^{-1} d. wt.

(iv) Nickel :- Nickel (Ni) is also an essential micronutrient for N_2 fixation in legume root nodules. It is an important component of hydrogenase, the enzyme responsible for recycling H_2 evolved as a by-product of nitrogen fixation (see Chapter 5). Nickel is involved in an oxidation reduction mechanism during hydrogenase catalysis (Eisbrenner and Evans, 1983); however, the nature of Ni binding to the hydrogenase protein remains to be determined. It has been shown that the derepression of hydrogenase in B. japonicum cells cultured in a highly purified medium was strikingly stimulated by the addition of $NiCl_2$ at 0.5µM concentration. Also, the hydrogenase activity in bacteroids from soybean nodules of plants supplied with Ni was at least 33% higher than the activity in bacteroids from plants lacking added Ni (Eisbrenner and Evans, 1983). It seems highly probable that increasing attention must be devoted to the requirement and biological role of Ni in the productivity of legumes and other N_2-fixing species.

(v) Zinc :- Marsh and Waters (1985a,1985b) studied the effect of zinc (Zn) nutrition on nodulation and N_2 fixation in cowpea under both field and green house conditions. They observed significant increases in nodule number, nodule dry weight and acetylene reduction when plants were supplied with $ZnSO_4$. The maximum

acetylene reduction was obtained when a pot having three plants was supplied daily with 200 ml of nutrient solution containing 3 ppm of $ZnSO_4$. This indicates that Zn addition enhances nodule growth and N_2 fixation.

Calcium and copper may also have some important role in nodule development and function; the requirement for these elements was observed to be more in the nodules than elsewhere in the plant (Robson, 1983). There are also reports which show that the addition of micronutrients such as iron, boron and manganese to the growing medium of legumes inoculated with Rhizobium have a marked effect on yield and dry matter (Demeterio et al, 1972; Kapur et al, 1975; Rhoden and Allen, 1982). However, from these studies it is not clear whether the increase in nodulation and N_2 fixation was the result of increased photosynthetic activity or of a direct effect of micronutrients on Rhizobium growth.

4.1.3. Temperature :

Root temperature has a marked effect on both development and N_2 fixing activity of legume root nodules (Pankhurst and Layzell, 1984). Both very low and very high temperatures are not conducive to the legume-Rhizobium symbiosis; nodulation is more sensitive to cold than nitrogenase activity (Sprent,1979). In Trifolium subterraneum, low root temperature (7°C) delayed Rhizobium infection (Roughley, 1970). On the other hand, at higher temperature (30°C) some strains of R. trifolii failed to differentiate into bacteroids and fix nitrogen in T. subterraneum (Pankhurst and Gibson, 1973). Other

studies have shown that considerable variability exists between legume species in the optimal temperature for N_2 fixation (Pankhurst and Sprent, 1976, Rennie and Kemp, 1981). Pankhurst and Layzell (1984) studied the effect of temperature on N_2 fixation in Lotus pedunculatus root nodules formed by one strain of R. loti and one strain of Bradyrhizobium. They found that the maximum nitrogenase activity in the root nodules formed by the strain of R. loti was at 12°C, while in the nodules formed by the strain of Bradyrhizobium was at 30°C. They also recorded that the rate of CO_2 evolution in these nodulated plants increased almost linearly with temperature. The increase in the respiration rate and decrease in nitrogenase activity with the rise in temperature resulted in a gradual rise in the apparent respiratory cost estimated on the basis of the ratio of CO_2 evolution: C_2H_2 reduction. Recently, Bertelsen (1985) studied the effect of temperature on H_2 evolution and acetylene reduction in pea nodules and in isolated bacteroids. With detached roots the relative efficiency calculated from acetylene reduction showed a decrease with a rise in temperature above 22°C. With excised nodules and isolated bacteroids similar results were obtained. However, the relative efficiency calculated from H_2 evolution in $Ar:O_2$ (80% Argon + 20% O_2) was unaffected by temperature.

4.1.4. Moisture

Nodulation and N_2 fixation are sensitive to both water stress and water-logged conditions. Worrall and Roughley (1976) reported that the moisture stress had an

inhibitory effect on T. subteraneum root hair infection. Gallacher and Sprent (1978) have examined the effect of moisture on nodule growth and nitrogenase activity in Vicia faba using both water-stressed and water-logged plants. They observed that water-stressed plants did not develop all those nodules which had been initiated. In their study waterlogging did not affect significantly the nodulation of V. faba. However, Minchin and Pate (1975) reported that water logging was unfavourable for nodule growth and N_2 fixation. Aparicio-Tejo and Sanchez-Diaz (1982) reported that in the root nodules of alfalfa the nitrate reductase (NR) activity increased but nitrogenase activity decreased gradually during drought, but on rewatering the NR activity decreased and nitrogenase activity increased quickly.

The question of whether water stress and water logging are unfavourable due to direct effects on the host plant, remains largely unanswered. Water stress might affect nitrogen fixation in several ways. Lower water potential in nodules may directly reduce nitrogen fixing activity with an accompaning reduction in nodule respiration (Pankhurst and Sprent, 1975); transport of fixed N out of nodules may be depressed (Minchin and Pate, 1975) or supplies of photosynthate from water-stressed shoot systems may be reduced (Huang et al,1975; Patterson et al,1979). Weisz et al (1985) studied the response of drought stress on N_2 fixation in soybean with in situ experiments performed during two seasons under different field conditions. They observed that drought

resulted in a decrease in acetylene reduction and nodule gas permeability. A short-term oxygen enrichment study demonstrated that nodule gas permeability might limit oxygen flux into both drought-stressed and well-watered nodules of these field-grown soybeans (Weisz et al, 1985). Nodules can recover from intermediate moisture stress on rewatering by resuming the impaired meristematic activity. Intermittent water logging reduces the oxygen supply to nodules and is therefore inhibitory to nitrogen fixation (Sprent, 1984).

4.1.5. Oxygen concentration :

Although the nitrogenase enzyme complex can be rapidly and irreversibly inactivated by exposure to oxygen (as discussed in chapter 2), the legume nodule requires oxygen to support the respiration associated with N_2 fixation. Intact nodules obviously have some means of protecting the nitrogenase enzyme complex from oxygen damage. Such protection mechamism may consist of leghaemoglobin (Appleby,1984) or some kind of diffusion barrier that might control the magnitude of oxygen diffusion into the bacteroid (Sheehy et al, 1983, 1985). Thus an optimum concentration of oxygen must be maintained in the bacteroid to avoid oxygen damage of nitrogenase (Patterson et al, 1983). At oxygen concentrations above ambient (1.0 atm of O_2 gas) the nitrogenase activity of nodulated soybean roots and also isolated bacteroids was observed to be greatly and irreversibly reduced (Patterson et al,1983).On the other hand, under the conditions of reduced O_2 supply the

nodule respiration and N_2 fixation activities were observed to decrease rapidly (Witty et al,1983; Ryle et al, 1984; Gordon et al, 1985). Ryle et al (1984) determined the rate of respiration of nodulated roots of soybean first in air (20% O_2) and then in 3% O_2 (0.034% CO_2, balance N_2). They observed that exposure of a nodulated root system to a gas stream containing 3% O_2 resulted in an immediate fall of respiration by 84% and complete inhibition of nitrogen fixation. Criswell et al (1976) also observed that exposure of nodulated root systems to 3% O_2 inhibited most N_2 fixation, although with short exposures the inhibition was reversible. Gordon et al (1985) estimated the respiratory efflux of $^{14}CO_2$ by nodules as a means of assessing the import of labelled photosynthate from leaves. $^{14}CO_2$ was supplied to the leaves; at the same time the root and nodule environment was exposed to air containing either 21% or 3% O_2 . They observed that very little $^{14}CO_2$ was respired from the nodulated root system exposed to 3% O_2 as compared to the nodulated root system exposed to 21% O_2. They also demonstrated that a reduction in O_2 level from 21% to 3% in the nodulated root system eliminated nodule metabolism and the bulk of $^{14}CO_2$ respiration. On the other hand changing the O_2 level back from 3% to 21% caused an immediate increase in the total $^{14}CO_2$ respiration. From these experiments it appears that the O_2 may be a limiting factor in the N_2-fixing nodules. Furthermore, under certain environmental conditions such as intermittent water logging, O_2 supply to the nodules may go severely below the optimum level and thus O2

supply may be the major rate-limiting factor for N_2 fixation under these conditions (Sprent, 1984).

4.1.6. Hydrogen evolution by nitrogenase:

It has been discussed in chapter 2 that H_2 is evolved as an inevitable by-product of N_2 fixation by nitrogenase. The extent of H_2 evolution from N_2-fixing legume root nodules depends on two factors: (i) the efficiency of electron use for N_2 reduction in the bacteroids, and (ii) the presence or absence of an efficient H_2 recycling system. In Rhizobium strains lacking an uptake hydrogenase system (Hup^-) the rate of H_2 evolution would depend only on the efficiency of electron use for N_2 reduction. H_2 evolution during N_2 fixation can be expressed as a function of the total energy flux through nitrogenase in terms of an electron allocation coefficient (EAC)[see chapter 2]. Even with N_2 as the substrate at physiological levels, at least one molecule of H_2 is produced for every molecule of N_2 reduced (ie at least two electrons are wasted for every six electrons used for N_2 reduction). Therefore the maximum possible EAC value in the presence of sufficient N_2 and a proper supply of reductant would be 0.75. But when N_2 is not available in sufficient amount or the supply of reductant to nitrogenase is low, more electrons merely recombine with protons evolving additional molecules of H_2. Thus the EAC value becomes less than 0.75. Rhizobium strains having an effective H_2 recycling system can recycle most of the H_2 evolved as the

by-product of N_2 fixation to regenerate energy in the form of ATP. The significance of H_2 recycling in N_2 fixation will be discussed in chapter 6.

The extent of H_2 evolution is reported to vary during the growth cycle of N_2-fixing legumes and to be influenced by environmental conditions. According to Bethlenfalvay and Phillips (1977) the percentage of electron flux lost as H_2 from pea root nodules changed from 60% during early stages of growth to about 30% for the period of 45 days to 68 days after planting. Drevon et al (1982) observed that an increase in the partial pressure of O_2 from 20% to 40% markedly increased the nitrogen activity and decreased by 16% the proportion of nitrogenase electron flux lost as H_2.

H_2 evolution is an energy expensive process in which both electrons and protons are wasted for no known benefit. Moreover, H_2 may act as a reversible inhibitor of N_2 fixation at the active site of nitrogenase reaction (Dixon, 1972; Dixon et al, 1981). Hup^+ strains which evolve little or no H_2 are considered superior to the Hup- strains, but hydrogen recyling by the Hup system may conceal underlying differences in the efficiency of the nitrogenase system itself. In other words a bacteroid operating with an electron allocation coefficient of 30% is likely to be less efficient than a system in which 75% of the electron flux is used for N_2 reduction, regardless of whether or not the released H_2 is recycled by the hydrogenase system.

4.2. Genetic factors:

Symbiotic N_2 fixation is the result of an interaction between the legume host and the Rhizobium microsymbiont. Therefore genetic variation either in the host or in the Rhizobium can affect symbiotic N_2 fixation. The establishment of particularly desirable combinations of host-microsymbiont may also be an important factor for genetic enhancement of symbiotic N_2 fixation.

4.2.1. Host genetic factors:

Only limited information is available on plant factors associated with N_2 fixation in legume nodules. Not all legumes can be induced to nodulate and fix N_2 by a single strain of Rhizobium. There is a limited host range for each Rhizobium species. In fact Rhizobium has been classified into different species according to their host specificity (Jordan and Allen, 1974).

In pea, nodulation in the cultivar "Afghanistan" provides a classical example of the effects of host on nodulation and N_2 fixation. The cultivar "Afghanistan" is not nodulated by most strains of R. leguminosarum that can form nitrogen-fixing nodules on other cultivars (Lie, 1978). Similarly in soybean, it has been reported that in most commercial cultivars the strains of R. fredii can not form nitrogen-fixing nodules; on cultivar 'Peking', however, these bacteria can form effective nitrogen fixing symbiosis (Keyser et al, 1982). Non-nodulating and ineffectively nodulating genotypes have been indentified in several legumes,

including alfalfa (Peterson and Barnes, 1981), peanut (Nigam *et al*, 1982) and chickpea (Davis *et al*, 1985). Another interesting example of the effect of host genotype on nodulation and N_2 fixation came from the studies of Jacobsen and Feenstra (1984) who developed a mutant of pea having nodulation and N_2 fixation ability even in the presence of 15 mM KNO_3. In soybean also, a supernodulation and nitrate-tolerant (*nts*) mutant has been recently reported (Carrol *et al*, 1985; Gresshoff *et al*, 1985). The mutant *nts*382 was developed by chemical mutagenesis with EMS of the commercial soybean cultivar Bragg. In the presence or absence of several combined nitrogen sources such as KNO_3, urea, NH_4Cl and NH_4NO_3, *nts*382 plants had considerably more nodules than wild type Bragg plants. The mutant character of *nts*382 is inherited as a Mendelian recessive factor. It is thus apparent from these studies that the host genotype can influence the symbiotic effectiveness of *Rhizobium* strains.

Reports on genetic variation in N_2 fixation associated with host plants have been reviewed recently by Cregan and Berkum (1984). They have suggested that qualitative genetic factors might be responsible for a proportion of the observed genetic differences in various legumes for nodulation and N_2 fixation. Seetin and Barnes (1977) and recently Heichel *et al* (1984) reported significant genetic variation in alfalfa cultivars for N_2 fixation potential. There is also evidence for the effect of host cultivar on uptake hydrogenase and

electron allocation coefficient for N_2 fixation (Bedmar et al, 1983; Lopez et al, 1983; Bedmar and Phillips, 1984a; Garg et al, 1985). Groat et al (1984) studied the host plant nodule enzymes associated with N_2 fixation in alfalfa and observed significant genetic variability in their relative amounts among the host cultivars.

Vincent (1980) suggested a number of characteristics of the legume host which may effect the symbiosis. The photosynthetic capability, the host-influence upon hydrogen evolution and the transport and assimilation of fixed nitrogen are characters of the host which may determine the physiological environment provided for N_2 fixation. Furthermore, the host is a major factor in determining nodule persistence and thus the duration of N_2 fixation (Vincent, 1980).

4.2.2. Rhizobium genetic factors:

Rhizobium strains which form effective root nodules on one legume species often form ineffective nodules that fix little or no N_2 on other legume species (Sloger, 1969; Pankhurst et al, 1979; Garg et al, 1985). Also genotypic differences are known to exist among different Rhizobium strains for their ability to fix N_2 on a particular host cultivar (El-Sherbeeny et al, 1977; Wynne et al, 1980; Nelson and Child,1981). El-Sherbeeny et al (1977) observed significant genetic differences amongst 20 field isolates of R. leguminosarum in their ability to fix nitrogen in V. faba. Large differences in the nitrogenase activity were reported in peanuts inoculated with a range of Rhizobium strains and grown in

the greenhouse as well as in the field (Wynne et al, 1980). Similarly Nelson and Child (1981) reported a wide range of variation in the N_2-fixing ability of 108 isolates of R. leguminosarum which were used to inoculate a Pisum sativum cultivar, grown under a controlled environment. Recently, Hattori and Johnson (1984) reported differences among several isolates of R. fredii (which form nitrogen-fixing nodules in soybean cultivar 'Peking') for the ability to nodulate and fix nitrogen in commercial soybean cultivars. They have demonstrated that one of the 5 isolates, USDA191 had a wider host range and was able to nodulate and fix nitrogen in several commercial cultivars as effectively as strains of B. japonicum. From these studies it is evident that the genotype of Rhizobium is an important factor controlling quantitative differences in N_2 fixation.

4.2.3. Host-Rhizobium combinations:

A particular host-microsymbiont combination may also be an important factor for increased N_2 fixation in a legume-Rhizobium symbiosis. Mytton et al (1977) inoculated six V. faba cultivars individually with six strains of R. leguminosarum. The variation due to the cultivar-strain combinations accounted for 73.8% of the total variation in plant dry weight. The large variation associated with the cultivar-strain combinations indicates the potential to enhance symbiotic nitrogen fixation and increase productivity through the use of

optimal host cultivar-*Rhizobium* strain combinations in agriculture. In a similar study, significant effect of cultivar-strain combinations for grain yield and nodule mass were detected in field grown chickpea (Rai and Singh, 1979). In peanut host cultivar-strain combination effects (interactions) for N_2-ase activity and total N were reported in a greenhouse experiment (Wynne *et al*, 1980) but similar interactions were not observed in a field study (Elkan *et al*, 1980). In a field-experiment Vest and Caldwell (1972) also reported no soybean host cultivar-strain combinations effect. These reports indicates that cultivar-strain combination effects, although frequently detected, are not observed in all cases. Such interactions may be more difficult to detect in the field than in the greenhouse where the population of *Rhizobium* and available soil N can be carefully controlled.

Chapter 5

BIOCHEMISTRY AND GENETICS OF HYDROGENASE

5.1 Introduction:

As discussed in chapter 4, the evolution of H_2 seems to be an unavoidable by-product of the nitrogen fixation reaction which is of no known benefit to the microorganism. N_2 fixing organisms including some strains of Rhizobium often possess a H_2-oxidising enzyme system called "uptake hydrogenase", that is capable of recycling the H_2 released as the by-product of nitrogenase activity. Although the uptake hydrogenase is a common feature among all nitrogen fixing bacteria and cyanobacteria, Rhizobium is unusual among diazotrophs in that, not all, but only some strains possess the hydrogen recycling (Hup) system. Many Rhizobium and Bradyrhizobium strains have been surveyed by different authors for the presence or absence of uptake hydrogenase and these have been summarised by Brewin (1984, see Table 5.1). From these surveys, it appears that the occurrence of hydrogenase in the root nodule bacteria is very rare particularly among the Rhizobium strains. Only 5-10% of the R. leguminosarum and R. meliloti strains have significant levels of hydrogenase activity. Similarly, in B. japonicum, only about 25% of field isolates possess hydrogenase activity. On the basis of theoretical estimates, such Hup^+ Rhizobium strains are expected to be more efficient in energy utilization for nitrogen fixation than Hup^- strains (Pate et al, 1981, see Table 3.1). The experimental observations and theoretical considerations

Table 5.1 Survey of *Rhizobium* strains for their ability to recycle hydrogen produced during nitrogen fixation (from Brewin, 1984)

Species of root nodule bacteria	Host legume	Number of strains examined	Number of Hup^+ strains*	Reference
A. *Fast growing***				
R. leguminosarum	... Pea	15	2	Ruiz-Argueso *et al*,1978
		108	12	Nelson and Child,1981
		48	2	Brewin, 1984
R. meliloti	... Alfalfa	19	4	Ruiz-Argueso *et al*,1979b
R. trifolii	... Clover	7	0	Ruiz-Argueso *at al*,1979b
R. phaseoli	... *Phaseolus vulgaris*	10	0	Brewin, 1984
B. *Slow growing*				
B. japonicum	... Soybean	1400	300	Lim *et al*, 1981
		32	7	Carter *et al*, 1978
Cowpea-*Bradyrhizobium*	... Cowpea	13	11	Schubert *et al*,1978
Mungbean-*Bradyrhizobium*	... Mungbean	30	28	Pahwa and Dogra, 1981

* Criteria for a Hup^+ strain vary slightly in different surveys, e.g. relative efficiency ≥ 0.8; significant levels of uptake of hydrogen or tritiated hydrogen by nodules or by bacteroids.

** Note that the specific activity for hydrogenase from bacteroids of any of the fast-growing rhizobia was less than 1% of the specific activity typical for bradyrhizobia.

concerning the possible benefits of hydrogen recycling system will be discussed in the next chapter. The present chapter is devoted to a description of the biochemistry and genetics of uptake hydrogenase in Rhizobium which will be useful in terms of assessing the physiological importance of hydrogenase. The current knowledge about hydrogenase of Rhizobium has come primarily from studies in B. japonicum, although some genetic studies have been made in R. leguminosarum also. Therefore, the biochemistry and genetics of hydrogenase will be examined with particular reference to B. japonicum .

5.2 Biochemistry of hydrogenase:

The hydrogenase enzyme is defined biochemically as catalyzing a reaction in which hydrogen yields two protons and two electrons. The B. japonicum hydrogenase from bacteroids has been purified under anaerobic conditions and extensively characterised in strains 110 (Arp and Burris, 1979) and 122DES (Arp,1985). In both cases bacteroids were prepared and broken by sonication. The particulate enzyme was solubilised by the treatment with Triton X-100 and further purified by polyethylene glycol fractionation and column chromatography. The specific activity of the enzyme was increased 196-fold to 19.6 unit per mg of protein. B. japonicum hydrogenase has also been isolated and purified from chemolithotrophically grown (ie grown on H_2, O_2 and CO_2) bacterial cells (Harker et al.,1984).

5.2.1 Biochemical properties of hydrogenase:

The biochemical properties of hydrogenase elucidated from the studies of Arp and Burris (1979) and

other authors are discussed below.

(a) Stability and storage:- In whole nodules frozen in liquid nitrogen, hydrogenase is stable for several months. The half life of purified hydrogenase, kept in an anaerobic condition, is several days at 4°C and about 5 hours at room temperature. The enzyme can be stored indefinitely in liquid nitrogen. The enzyme is oxygen sensitive with a half life of 70 minutes when exposed to air at room temperature.

(b)Relative molecular mass:- From earlier studies of Arp and Burris (1979), the hydrogenase enzyme appeared to be a monomer of relative molecular mass of about 64,000. Stults et al (1984) also suggested that the enzyme isolated from B. japonicum cells derepressed for hydrogenase was a monomer. However, in recent studies of Arp (1985), the relative molecular mass of hydrogenase was found to be 104,000 as determined by sucrose density gradient centrifugation. Electrophoresis in the presence of sodium dodecyl sulphate revealed two subunits with relative molecular mass of 64,000 and 35,000, indicating an $\alpha\beta$ subunit structure. Harker et al (1984, 1985) also demonstrated that the hydrogenase from chemolithotrophically grown cells of B. japonicum was a dimer with polypeptides of relative molecular mass 60,000 and 30,000. The antiserum raised against the 60 KD polypeptide did not cross-react with with the 30 KD polypeptide indicating that the 30 KD protein was a unique polypeptide of hydrogenase and not a breakdown product of the larger protein (Harker et al 1984,).

(c) pH profile:- The optimum pH is 5.3 for reduction of methylene blue, 5.7 for reduction of ferricyanide. Particulate hydrogenase from soybean bacteroids takes up H_2 optimally near pH 9.0 but the optimum shifts to near 5.3 upon solubilization.

(d) Acceptor specificity:-The purified enzyme does not take up H_2 with O_2 as the electron acceptor. It does, however, rapidly transfer electrons to methylene blue, ferricyanide cytochrome C, 2,6 dichlorophenol indophenol but not to NADP, FAD or FMN (Arp and Burris, 1979).

(e) Amino acid composition:- The amino acid composition of the protein revealed a high percentage of hydrophobic amino acids which is consistent with the particulate nature of the protein (Arp, 1985). About 20 cysteine residues per molecule were indicated by performic acid oxidation of the protein.

(f) Absorption spectra of hydrogenase:- The absorption spectrum of hydrogenase was found to be a broad absorption band in the 400 nm region with a peak at 409 nm (Arp, 1985).

(g) Metal content:- Analysis of metal content indicated 0.59±0.06 mol Ni and 6.5±1.2 mol Fe per mol hydrogenase (Arp, 1985). Hydrogenase from chemolithotrophically cultured B. japonicum has been characterised as a nickel enzyme (Harker et al, 1984). The presence of Ni in rhizobial hydrogenase was suggested from growth studies of B. japonicum (Klucas et el,1983). When cells were placed under chemolithotrophic growth condition in the absence of Ni, no growth occurred and no hydrogenase activity was present. When Ni was included in the medium, growth

occurred and hydrogenase activity was also present. The absence of Ni in the nutrient solution from soybean plants also resulted in decreased hydrogenase activity in bacteroids (Klucas et al, 1983).

(h) Immuno diffusion:- Antisera to rhizobial hydrogenase generated in a rabbit cross-reacted with the enzyme in all stages of purification (Arp, 1985). However, neither broken cells nor Triton treated membranes of Alcaligenes eutrophus gave a cross-reaction with antisera to the rhizobial hydrogenase (Arp, 1985).

5.2.2 Electron transfer pathway:

It has been well established that O_2 is the final acceptor for electrons for uptake hydrogenase system from R. leguminosarum (Dixon, 1968) and B. japonicum (Schubert et al, 1977; Carter et al,1978). However, O_2 does not react directly with hydrogenase, but electrons from H_2 are transferred via a series of electron carriers to O_2. How the intermediate electron carrier components are involved in the hydrogenase reaction is discussed below.

The first electron acceptor from hydrogenase might be a b-type cytochrome, identified and named as component 559-H_2 in Hup^+ strains of B. japonicum (Eisbrenner and Evans, 1982b). The cellular concentrations of component 559-H_2 was positively correlated with the level of hydrogenase activity for a range of strains. This correlation indicates that component 559-H_2 may be an electron carrier that couples hydrogenase to the main electron pathways of Rhizobium

(Eisbrenner and Evans, 1982b). Subsequent to the component 559-H_2, the next intermediate component involved in the chain of electron carrier might be ubiquinone (Eisbrenner and Evans, 1982a), because dibromothymoquinone, a ubiquinone antagonist, inhibited O_2 dependent H_2 uptake by bacteroids. The involvement of cytochrome b and c has also been implicated, because when endogenous respiration was inhibited by iodoacetate or malonate, the addition of H_2 resulted in a marked increase in the rate of cytochrome reduction (Eisbrenner and Evans,1982a; Eisbrenner et al, 1982). Finally a cyanide sensitive terminal oxidase, cytochrome a-a_3 is involved in the transfer of electrons to O_2 (Ruiz-Argueso et al, 1979a). It is probable that all these components, ubiquinone to a-a_3 are common to the two electron transport pathways from hydrogen and from other substrates.

Thus, biochemical studies provide evidence that at least three polypeptides are needed for the hydrogenase system to function effectively within nodule bacteroids, namely the two hydrogenase polypeptides and the component 559-H_2.

5.3 Genetics of hydrogenase:

Our present knowledge about the genetics of uptake hydrogenase of Rhizobium came from the studies made with B. japonicum strain 122DES and R. leguminosarum strain 128C53. The various genetic studies involving localization, mutagenesis and cloning of the hydrogenase genes are summarized below.

5.3.1 Localization of hup genes:

It has been established that the determinants for several symbiotic genes (eg nod, nif) are located on large plasmids in the fast growing Rhizobium species (Beringer,1980; Brewin et al,1981; Denarie et al 1981; Kondorosi et al,1981). Brewin et al (1980) demonstrated that the hup genes in R. leguminosarum strain 128C53 was contained in a plasmid, pRL6JI. This plasmid also determines nodulation (nod), host-range and N_2 fixation (fix). The transfer of these markers to a Hup- strain of R. leguminosarum was associated with the co-transfer of the hydrogenase activity (Brewin et al,1980, 1981, 1982). Similarly, in another Hup^+ field isolate of R. leguminosarum strain 18A from Denmark, the hup determinants are located on the symbiotic plasmid (Brewin 1984). Although, the studies with R. leguminosarum indicate that determinants for hydrogenase are plasmid borne, an examination of B. japonicum strains revealed no evidence of plasmids carrying genes for H_2 uptake activities (Cantrell et al,1982). Earlier, Cantrell et al (1981) postulated that hydrogenase determinants in Hup^+ B. japonicum strains were either present on the chromosome or alternatively, they might be carried on a plasmid that was too large to be recovered by standard physical techniques. Obviously, this area requires further study. It should be noted that in Bradyrhizobium strains, such as B. japonicum, the nitrogen fixation (nif) genes are also probably not on plasmids (Haugland and Verma,1981; Masterson et al,1982).

5.3.2 Isolation of Hup- mutants:

In order to assess the role of hydrogenase in the legume-Rhizobium symbiosis, mutants must be isolated and their effects on symbiosis determined. In B. japonicum hydrogenase mutants have been isolated by screening bacteria on agar in the presence of hydrogen for the inability either to reduce the dye methylene blue or to grow chemolithotrophically (Lepo et al,1981; Maier,1981) both of which activities require hydrogenase. In R. leguminosarum such screening of bacteria is not possible because the hydrogenase appears to be expressed only in the nodule and not in the free living state. Therefore, screening for hydrogenase mutants must be done on plants, where the bacteria differentiate into the nitrogen fixing bacteroid forms.

In B.japonicum various classes of Hup- mutants have been isolated. Revertible mutants that affect hydrogenase expression have been isolated in B. japonicum by Lepo et al (1981). They mutagenised the strain "SR", a streptomycin and kanamycin resistant derivative of the Hup^+ B. japonicum strain USDA122DES, using nitrous acid. By screening clones that were chemolithotrophy-negative, they isolated two Hup- mutants, PJ17 and PJ18, from which Hup^+revertants, PJ17-1 and PJ18-1 were subsequently isolated. Bacteroids isolated from PJ17 and PJ18 have both been examined by two dimensional gel electrophoresis and shown to lack a protein which is probably the 64 KD hydrogenase polypeptide (Drevon et al, 1982). In addition, bacteroids of both mutants lacked the spectral

signals normally associated with component 559-H_2 after exposure to hydrogen (Eisbrenner and Evans, 1982b), a result that would be expected if hydrogenase was required for the H_2 dependent reduction of component 559-H_2. Mutant strains of B. japonicum unable to grow chemolethotrophically with H_2 have also been reported by Maier (1981). He classified these mutants into several different groups. These included strains unable to oxidize H_2 (Hup-) and strains lacking ribulose 1-5 biphosphate carboxylase activity (Maier, 1981). Some other mutant strains were able to oxidise H_2 as bacteroids from soybean nodules but in free living culture required lower oxygen levels than the wild type parent strain for the expression of the H_2 uptake systems (Maier and Merberg, 1982). Maier and Mutaftschiev (1982) conducted an in vitro complementation experiment to reconstitute H_2 uptake activity by mixing together extracts from bacteroids of two different Hup- mutants. In this way, they were able in one case to demonstrate biochemical complementation between two chemolithotrophy-negative mutations. Both the range of mutant phenotypes and the observation of in vitro complementation suggest that several different genes may be involved in the hup system.

Merberg and Maier (1983) isolated several mutants by various ways in B. japonicum strain "SR" that were able to grow chemolithotrophically in atmosphere containing 10% oxygen, a condition in which wild type strain can not grow. These mutants contain more

hydrogenase than the parent strain both as free living cells and as bacteroids in N_2 fixing soybean root nodules.

Kagan and Brewin (1985) mutagenised the R. leguminosarum strain 128C53 using the transposon "Tn5-mob"on the suicide vector pSup5011 and isolated eight Hup- mutants. Physical and genetic analyses of these Hup- mutants suggest that each mutant was due to a single insertion of Tn5-mob into the symbiotic plasmid pRL6JI (see Fig 5.1)

5.3.3. Cloning hup genes:

In order to understand the hup genes at the molecular level, it is necessary to clone the Hup specific DNA sequences. Cantrell et al (1983) made a gene bank in E. coli from the total DNA of the Hup^+ B. japonicum strain 122DES in the broad host range cosmid vector pLAFR1. The gene bank in E. coli was mated with the revertible Hup- mutant strain PJ17. The transconjugants were screened for Hup^+ phenotype on the basis of the ability to grow chemolithotrophically and to reduce methylene blue. Hup^+ transconjugants were isolated from the gene bank at a frequency of $6x10^{-3}$ per recipient. These Hup^+ transconjugants were found to carry cloned DNA fragments that restored the Hup- mutant PJ17 to a Hup^+ phenotype in both free-living and bacteroid forms. DNA from eleven of the cosmid clones that suppressed PJ17 was compared after digestion with a restriction enzyme. All the clones contained a common DNA region of 18.2 kilobases in length. Presumably this common region contains the cloned wild type copy of the gene that is mutated in

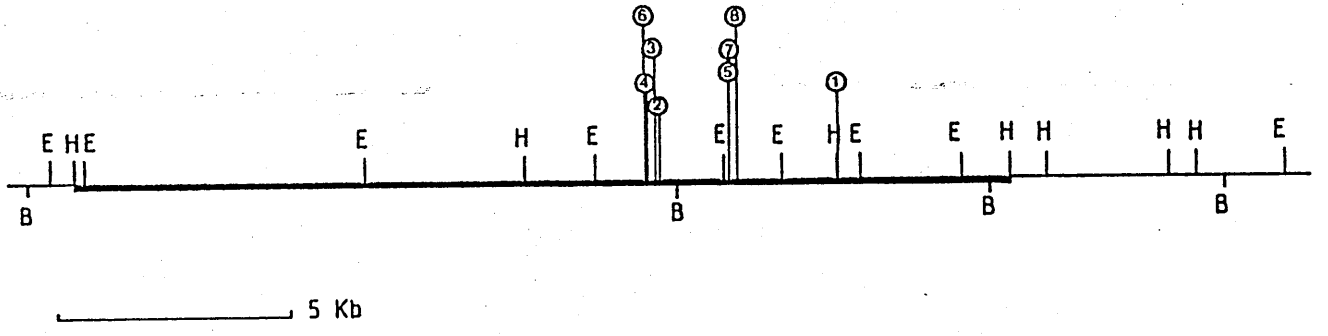


Fig 5.1. Restriction map of hup region of pRL6JI of R. leguminosarum that has been cloned in pLAFR1 by Dr A. Krol (Amsterdam). H : HindIII, B : BamH1, E : EcoR1. The numbers 1-8 indicate the positions of Tn5mob insertion in the eight Hup$^-$ mutants isolated by Kagan and Brewin (1985). The thicker line indicates the region which hybridizes with hup DNA from B. japonicum cloned in pHU1. (A. Krol, 1986; submitted to MGG)

PJ17. One of the cosmids named pHU1 containing the 18.2 kilobases common hup DNA region has been studied further in more details (Haugland et al, 1984). Analysis of site directed Tn5 insertions into the 122DES genome indicates that Hup specific sequences occur in a region spanning about 15 kilobases of insert DNA within pHU1. Genetic complementation analysis indicates that the hup genes in B. japonicum strain 122DES appeared to be organized in at least two, and probably three transcriptional units (Haugland et al, 1984).

The cloned DNA in pHU1 could suppress several other revertible Hup- mutations besides PJ17. The eight Hup- mutants of R. leguminosarum strain 128C53 isolated by Kagan and Brewin (1985) were suppressible by pHU1. However, there are other Hup- mutations, which can not be suppressed by pHU1. Two Hup- mutants of R. meliloti, SR3 and USDA16 could not be complemented by pHU1 (Haugland et al,1984). Some of the Hup- mutants, both revertible and non-revertible could be complemented by pHU1 only at a low frequency (Haugland et al,1984). One such revertible Hup- mutant PJ18 (Lepo et al, 1981) of B. japonicum was found to be suppressed by pHU1 at a frequency of one colony per thousand. This indicates that mutation in PJ18 might be dominant. In this event, Hup^+ pHU1 transconjugants of PJ18 would be obtained when sequence exchange caused by double reciprocal recombination between the cosmid and the PJ18 genome was followed by segregational loss of the cosmid containing the mutant sequence (Haugland et al, 1984).

Recently, Lambert et al (1985) have isolated four new cosmid clones containing additional hup genes by conjugal transfer of a B. japonicum 122DES gene bank into a Tn5-generated Hup- mutant and screening for additional acquisition of Hup activity. The newly isolated cosmids, pHU50-pHU53, contain part of the previously isolated pHU1 but extend as far as 20 kilobases beyond its border. One of these cosmid clones, pHU52, confers Hup activity on several Hup- wild type B.japonicum strains in free-living state and in nodules. Transconjugants obtained from interspecies transfer of pHU52 to certain R. meliloti and R. leguminosarum strains showed hydrogen dependent autotrophic growth (chemolithotrophic) by virtue of the introduced genes. pHU52 contained a 5.5 kb additional fragment not found in pHU1 (see Fig 5.2).

Hom et al (1985) recently have isolated a few recombinant cosmid clones containing a B. japonicum gene involved in both hydrogenase (Hup) and nitrogenase (Nif) activities. A B. japonicum gene bank utilizing broad host-range cosmid, pLAFR1 was conjugated into a $Hup^{-}Nif^{-}$ B. japonicum strain, SR139. Transconjugants containing the nif/hup cosmid were identified by their ability to grow autotrophically with hydrogen. One nif/hup recombinant cosmid also complemented several $Hup^{-}Nif^{+}$ mutants . This suggests that this cosmid contains a nif/hup gene as well as some other hup genes. The cloned DNA in this nif/hup cosmid clone was approximately 23 kilobases and contained 4 EcoR1 fragments of size 13.2, 4, 3 and 2.5 kilobases.

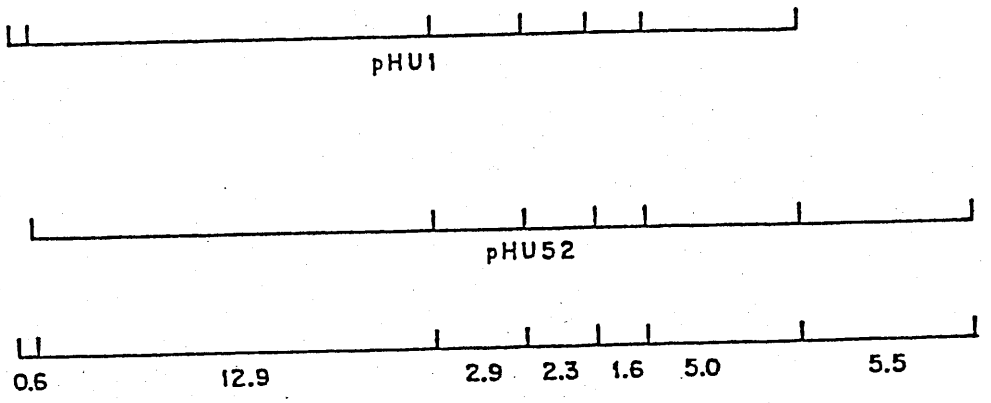


Fig 5.2. EcoR1 restriction map of cosmids pHU1 and pHU52 containing cloned hup DNA from B. japonicum. EcoR1 fragment sizes are in kilobase pairs. (Adopted from Lambert et al (1985).

5.4. Host genetic factors affecting hydrogenase activity:

Both plant and bacterial factors can affect H_2 evolution from legume root nodules. Dixon (1972) showed that R. leguminosarum strain ONA311 expressed strongly Hup^+, slightly Hup^+ or Hup^- phenotypes on Pisum sativum, Vicia bengalensis and V. faba respectively. Keyser et al (1982) reported two B. japonicum strains which were Hup^+ on three cowpea cultivars but Hup^- on three soybean cultivars. Garg et al (1985) also observed host dependent expression of uptake hydrogenase in different legumes of cowpea group. Such observations in different species of legumes are convincing evidence for host plant control of Hup phenotype. One interesting example of the effect of host cultivar within a species on the expression of Hup activity came from the studies of Bedmar et al (1983), who observed that the R. leguminosarum strains 128C53 and 3960 were Hup^+ on cultivar JI1205, moderately Hup^+ on Alaska and Hup^- on Feltham First. In another study Bedmar and Phillips (1984a) recorded a large variation in Hup activity of R. leguminosarum strain 128C53 with nine cultivars of pea. Recently Bedmar and Phillips (1984b) studied Hup activity on plants produced by different combinations of shoot and root grafting from three cultivars namely JI1205, Alaska and Feltham First, which showed different levels of Hup activity in symbiosis with R. leguminosarum strain 128C53 in a previous study by Bedmar et al (1983). Graft between JI1205 or Alaska and Feltham First in either root/shoot combination resulted in an increased Hup

activity over 'Feltham First/Feltham First' combination of shoot/root graft in symbiosis with strain 128C53. The combination of shoot/root graft in which JI1205 or Alaska was used as shoot on Feltham First showed more hydrogen uptake activity in the nodules than the reverse combinations. These results show that both shoot and root genotypes of the host can influence the expression of Hup activity in the nodules. The mode of inheritance of the host genes involved in the expression of Hup activity in legume root nodules is not yet reported.

5.5. Conclusions:

The above discussion summarises our current knowledge about the biochemistry and genetics of uptake hydrogenase in Rhizobium. An understanding of the biochemistry and genetics of hydrogenase will help us to assess the role of hydrogenase in symbiosis. Both the biochemistry and the genetics indicate that the hydrogen recycling system is unexpectedly complex. Mutagenesis and gene cloning experiments suggest that a region of about 21 kilobases of DNA is essential for hydrogenase activity (out of this 21 kb region about 16 kilobases are common to both pHU1 and pHU52). This is equivalent in size to the complete nitrogenase (nif) region from Klebsiella. Biochemical functions studied have indicated that at least two polypeptides are present in the hydrogenase enzyme itself; there is also a nickel and iron component and spectroscopic data indicate the presence of at least one new electron transport factor in the cytochrome chain

The isolation and characterization of revertible Hup- mutants of Rhizobium are of special significance, because their use in genetic analysis will help us to examine the potential benefits of the hydrogenase system in symbiosis by comparing isogenic strains, that differ only for the presence or absence of a functional hydrogenase system. For this purpose revertible Hup- mutants of Rhizobium have been compared to the Hup^+ parent strains and also to Hup^+ revertant strains in order to correlate hydrogenase activity with improved energy efficiency in symbiosis. (These will be discussed in the next chapter.) Alternatively, Hup^+/Hup^- comparisons could be made after introduction into a Hup- strain of cloned DNA carrying determinants for a complete hydrogenase system. Studies involving such comparisons will be discussed in the next chapter.

Chapter 6

PHYSIOLOGICAL SIGNIFICANCE OF UPTAKE HYDROGENASE IN THE LEGUME-*RHIZOBIUM* SYMBIOSIS

6.1. Introduction:

It has been discussed in the last chapter that only some strains of *Rhizobium* possess an uptake hydrogenase system. This raises the question whether such Hup^+ strains are more efficient in their nitrogen fixing ability than Hup- strains. Theoretical estimates, discussed in chapter 3, indicate that Hup^+ strains should be superior to the Hup- strains in terms of energy utilization for N_2 fixation. Therefore possession of an uptake hydrogenase system, which can recycle H_2 evolved as a by-product of N_2 fixation is expected to confer certain benefits to the N_2-fixing process. If energy is the major limitation for N_2 fixation in legumes, then coupling of the oxidation of H_2 to energy-yielding processes conceivably could increase the rate or the efficiency of N_2 fixation. Alternatively, the conservation of energy through H_2 recyling might decrease the demand for photosynthate and consequently contribute to increased dry matter production. Pate *et al* (1981) showed theoretically that the cost of N_2 fixation in Hup^+ strains of *Rhizobium* is considerably less than in Hup- strains (see Table 3.1 in chapter 3). Similarly, Evans *et al* (1981) predicted that soybean seedlings inoculated with a Hup^+ strain of *Bradyrhizobium* and grown for a period of 40 days would show a 21% increase in dry weight

compared to plants in which H_2 recycling did not occur.

Besides conserving energy in the form of ATP, the hydrogenase system might provide a mechanism to remove both H_2 and O_2 from the active site of nitrogenase where they might act respectively as reversible and irreversible inhibitors of N_2 fixation (Dixon, 1972). Recently, Dixon et al (1981) have presented a calculation based upon a mathematical model which is consistent with the conclusion that the hydrogen concentration inside pea and lupin root nodules may reach a concentration sufficient to inhibit nitrogenase activity. They argue therefore that the removal of inhibitory H_2 in nodules would be a major benefit of uptake hydrogenase system.

Moreover, it is known that Hup^+ strains of B. japonicum can grow as chemoautotrophs (Lepo et al,1980). Also, very recently Lambert et al (1985) have found that certain R. meliloti and R. leguminosarum strains containing cloned hup genes from B. japonicum in recombinant plasmids showed hydrogen-dependent autotrophic growth by virtue of the introduced genes. These findings raise another interesting possibility,namely that hydrogenase is of importance to the bacterium in some ecological niches in the soil. Eisbrenner and Evans (1983) suggested one possibility that H_2 and CO_2 evolved from a legume root nodule occupied by a Hup- Rhizobium strain could provide the necessary energy and carbon sources for hydrogen-dependent autotrophic growth of a Hup^+ Rhizobium strain on the surface of that nodule.

In view of the above theoretical possibilities, it

is important to examine critically the experimental observations reported in the literature in order to ascertain if uptake hydrogenase provides any real benefit in the legume-*Rhizobium* symbiosis.

6.2. Hypothesis and definations:

A number of experiments have been conducted in order to evaluate the physiological significance of uptake hydrogenase in symbiotic N_2 fixation. These experiments are all designed to test the following working hypothesis.

Hypothesis: " A functional hydrogenase system in *Rhizobium* strains results in a detectable improvement in the symbiotic performance by a host legume under controlled environmental conditions."

Before discussing particular experiments, the various keywords of this hypothesis such as "symbiotic performance", "*Rhizobium* strains", "host legume", "environmental conditions" and "detectable improvement" will be defined and explained below. With these definitions in mind it will be possible to analyse various experimental results in the light of this hypothesis.

Symbiotic performance: Any enhancement in the plant performance in the form of dry matter, seed yield or N content due to plant-*Rhizobium* association would be referred to as an improvement in the symbiotic performance. Nitrogenase activity in the root nodule is often considered as indicative of the level of symbiotic

performance. Symbiotic performance of Rhizobium strains with the host legume is most commonly measured as the rate of nitrogenase activity in nodules deduced from the acetylene reduction assay. However, observations on both attached nodulated roots and detached nodules have revealed that nitrogenase activity in many legume species declines rapidly in the presence of acetylene (Minchin et al, 1983). Minchin et al (1982) noted that the cumulative curve of ethylene production was non-linear over a 30 or 60 min assay period. Therefore, calculations of nitrogenase rate based on cumulative ethylene production will underestimate actual rates and produce low apparent values for N_2 fixation. On a longer time-scale, symbiotic nitrogen fixation in legumes is often estimated through $^{15}N_2$ enrichment by exposing the nodulated root system to $^{15}N_2$ gas. Measurements of the amounts of nitrogen obtained through symbiotic nitrogen fixation can also be determined indirectly by measuring seed yield, total dry matter and nitrogen content of plants inoculated with Rhizobium strains and comparing them with uninoculated plants grown in nitrogen-free medium. Another way of comparing symbiotic performance of Hup^+ and Hup^- nodules would be to compare nodule respiration. It is expected that CO_2 respired by Hup^+ nodules will be less than by Hup^- nodules.

Rhizobium strains:- In order to assess the significance of the Hup system, it is necessary to compare the symbiotic performance of different Hup^+ and Hup^- Rhizobium or Bradyrhizobium strains. Several kinds

of comparisons involving different Hup^+ and Hup^- strains are possible, such as:

i) Legumes inoculated with groups of randomly selected Hup^+ and Hup^- wild-type strains. This kind of comparison between groups of Hup^+ and Hup^- isolates is based on the assumption that genetic variation among all strains is random. However, comparison of single strains or small groups of Hup^+ and Hup^- strains are open to the criticism that genetic differences other than hydrogenase may influence the performance.

(ii) Symbiotic performance of Hup^+ parental strains and Hup^- revertible mutants: Theoretically, a comparison of Hup^- mutant strains with their Hup^+ revertants or with the wild-type parents as inoculants should be ideal for the evaluation of the possible advantages of H_2 recycling capability towards symbiotic N_2 fixation.

(iii) Comparisons involving any Hup^- strains with corresponding Hup^+ derivatives containing hup genes introduced as part of a recombinant plasmid: Interpretations from such comparisons may sometimes become difficult when the recombinant plasmid is unstable.

Host legume:- It has been discussed earlier (see 4.2.1) that hydrogenase activity of certain Rhizobium strains depends to a large extent on host species and host cultivars Therefore appropriate host species and cultivar must be used for comparison of hydrogenase activity in Rhizobium strains. Moreover, because of the

range of nodule morphology (eg, determinate or indeterminate), nodule physiology (ureide or amide transporters) and nodule occupancy (fast or slow growing Rhizobium strains), it may never be possible to make generalizations about the value of the hydrogen recycling system under all symbiotic conditions.

Environmental conditions:- In order to compare the symbiotic performance of Rhizobium strains, experiments must be conducted under specific environmental conditions ideal for both plant growth and symbiotic N_2 fixation. Specific reproducible environmental conditions can be maintained in growth cabinet or glass houses. In the growth cabinet and glass houses the environmental conditions such as temperature, humidity, light intensity and duration etc can be steadily maintained and plants can be grown without contamination from any soil bacteria. In field experiments the conditions are variable and contamination from pre-existing soil rhizobia is almost unavoidable. Therefore, the results from field experiments are very difficult to interpret.

Detectable Improvement: It is often difficult to detect slight variation in symbiotic performance due to different Rhizobium strains. In order to minimize experimental error and to increase the precision of statistical comparison it is desirable to keep many randomised replicates and to grow plants in identical environmental regimes in which only the nature of the

inoculant strains is variant. In most statistical comparisons significance levels at 5%, 1% or both are assessed.

6.3. Review of experimental results:

The experiments reported in the literature involved in assessing the possible effect of hydrogenase in symbiosis can be broadly classified into three groups:

(i) Experiments comparing groups of Hup^+ and Hup^- strains.

(ii) Experiments comparing Hup^+ and Hup^- isogenic strains.

(iii) Experiments comparing Hup^- strains with genetically engineered Hup^+ derivatives.

The various experiments are discussed below. In this discussion the results for slow-growing and fast-growing rhizobia are presented separately wherever possible.

6.3.1. Comparison of Hup^+ and Hup^- strains:

The advantages of hydrogen recycling system as proposed by Dixon (1972) and Dixon et al (1981) have been experimentally examined in several Rhizobium-legume associations. Such experiments where the symbiotic performances of Hup^+ and Hup^- strains have been compared, are summarised in Table 6.1. Schubert et al (1978) tested whether the presence of hydrogenase increases the efficiency of N_2 reduction, leading either to increased N_2 fixation or decreased respiratory cost. In this study

Table 6.1. Comparison of Hup^+ and Hup^- strains

Comparison of *Rhizobium* strains	Experimental conditions	Host legume	% increase*[1] dry matter	% increase total N	Reference
Slow growing					
1. (a)One Hup^+ and one Hup^- strains of *B. japonicum*	Controlled environment	Soybean	24.0	31.0	Schubert *et al* (1978)
(b)One Hup^+ and one Hup^- strains Cowpea-*Bradyrhizobium*	Controlled environment	Cowpea	11.0	15.0	Schubert *et al* (1978)
2. One Hup^+ and one Hup^- strains of *B. japonicum*	Green house	Seven soybean cultivars	11.6-106.6	NT*	Carter *et al* (1978)
3. (a) 5 Hup^+ and 5 Hup^- strains of *B. japonicum*	Growth cabinet	Soybean	15.7	26.2	Albrecht *et al* (1979)
(b) 3 Hup^- mutants and the Hup^+ parental strains of *B. japonicum*	Growth cabinet	Soybean	32.0	49.0	Albrecht *et al* (1979)
4. One Hup^+ strain "SR" of *B. japonicum* and one Hup^- mutant derived from SR	Controlled environment	Soybean	27.0	22.0 (seed)*[2]	Zablotowicz *et al* (1980)
5. 6 Hup^+ and 4 Hup^- wild type straind and 2 Hup^- mutant strains of *B. japonicum*	Field experiment	Soybean	NT	8.4	Hanus *et al* (1981)
6. Wild type Hup^+ and Hup^- strains of *B. japonicum*	controlled environment	soybean	NS*	NS	Gibson *et al* (1981)

/continued

Table 6.1 (continued)

Comparison of *Rhizobium* strains	Experimental conditions	Host legume	% increase*$_1$ dry matter	%increase total N	Reference
7. 28 Hup$^+$ and 2 Hup$^-$ field isolates and a group of Hup$^-$ mutants of Mungbean-*Bradyrhizobium*	Controlled environment	Mungbean	13-56	21-46	Pahwa and Dogra (1981)
8. One Hup$^+$ and one Hup$^-$ strains of Cowpea-*Bradyrhizobium*	Controlled environment	Cowpea	NS	NT	Rainbird *et al* (1983)
Fast growing					
9. Groups of low and high H_2 evolving strains of *R. leguminosarum*	Green house	Pea	NS	NT	Ruiz-Argueso *et al* (1978)
10. Two Hup$^+$ and three Hup$^-$ strains of *R.leguminosarum*	Controlled environment	Pea	NS	NS	Bethlenfalvay and Philips (1979)
11. Groups of wild type strains of *R. leguminosarum*	Growth cabinet	Pea	NS	NS	Nelson and Child (1981)
12. Groups of *R. leguminosarum* strains	Controlled environment	Pea	NS	NS	Truelsen and Wyndaele (1985)

*NT-not tested; NS- non-significant; *$_1$- % increases in total dry matter and total N content of plants inoculated with Hup$^+$ strain compared to those inoculated with Hup$^-$ strain; *2- indicates N content of seed.

the total dry matter and total N content during the vegetative growth of soybeans inoculated with one Hup^+ and one Hup^- B. japonicum strains were compared. In a similar comparison they inoculated cowpea with one Hup^+ and one Hup^- cowpea-Bradyrhizobium strains. The results of these experiments suggested that inoculation with Bradyrhizobium strains capable of recycling H_2 might result in gains in total N as well as total dry matter. However, these results could be criticized for two reasons: (i) each comparison was made with only two strains and (ii) the strains were not isogenic. Similarly, Carter et al (1978) used one Hup^+ and one Hup^- B. japonicum strain to inoculate seven soybean cultivars grown under controlled environmental conditions in the greenhouse. They observed 11.6 to 106.6% increase in the dry weight of plants with the Hup^+ strain over those inoculated with the Hup^- strain. However, these results are questionable for two main reasons: (i) the two strains were not isogenic and (ii) the data for each replicate were recorded on two plants only; thus the differences observed may partly be due to sampling error.

Albrecht et al (1979) conducted two experiments under a controlled environment in growth chamber to compare the symbiotic performance of Hup^+ and Hup^- B.japonicum strains with soybean plants. In the first experiment they inoculated five Hup^+ strains and five Hup^- strains and observed significant increases in dry weight and total nitrogen in plants inoculated with Hup^+

strains (Table 6.1). In the second experiment they compared the symbiotic performance of three Hup^- mutants with the parental Hup^+ strain "SR". In comparison with the plants inoculated with the Hup^- mutant, those plants inoculated with the Hup^+ parental strain recorded significantly higher dry weight and total N content (Table 6.1). Although these results demonstrated the superiority of Hup^+ *B. japonicum* strains, these can be criticized for the following reasons: i) the groups of five Hup^+ and five Hup^- strains in the first experiment are not big enough to assume that the genetic variability, independent of the hydrogenase phenotype, is random among these Hup^+ and Hup^- strains, and ii) in the second experiment the isogenicity of the mutants and parent strain has not been established. The Hup^+ strain SR and one of its Hup^- non-revertible mutants SR3 (Albrecht *et al*, 1979) have been further studied for their symbiotic performance on soybean in an another experiment by Zablotowicz *et al* (1980). They found 27% increase in dry matter and 22% increase in total nitrogen content of seeds of plants inoculated with Hup^+ strain SR as compared to plants inoculated with the Hup^- mutant SR3.

Hanus *et al* (1981) compared 6 Hup^+ and 4 Hup^- wild type strains and 2 Hup^- mutant strains of *B.japonicum* for the symbiotic performance on soybean grown on fields at four locations. In all locations, plants inoculated with Hup^+ strains appeared greener and produced seed with a significantly higher percentage nitrogen content than

plants inoculated with Hup^- strains. Average data from the experiments at four locations showed that Hup^+ inoculants increased crude seed protein by 8.9%. Thus this result indicated that Hup^+ strains of B. japonicum are significantly better inoculants than Hup^- strains ($p < 0.01$ in three experiments and $P < 0.05$ in one experiment).

In contrast to the above results, Gibson et al (1981) observed no significant increase in dry weight and total nitrogen of soybean plants inoculated with Hup^+ strains of B. japonicum under controlled environment. However, these results are also inconclusive for the main reason that the exact number of strains used in this comparison and their sources are not known.

Comparison of Hup^+ and Hup^- strains have also been reported in other bradyrhizobia, such as mungbean-Bradyrhizobium and cowpea-Bradyrhizobium. Pahwa and Dogra (1981) compared the nitrogen fixation efficiency of three sets of mungbean-Bradyrhizobium, namely a group of 28 Hup^+ field isolates, 2 Hup^- field isolates and a group of Hup^- mutants isolated from the wild type Hup^+ strain M28 by chemical mutagenesis. Mungbean plants inoculated with different Bradyrhizobium strains were grown under controlled environment in the greenhouse. There were 13-56% increases in dry matter and 21-46% increases in the total nitrogen of plants inoculated with Hup^+ isolates compared to the plants inoculated with the two Hup^- isolates. In the second comparison involving Hup^- mutants with the wild type Hup^+ parental strain, there

were 19-22% decreases in dry weight and 20-26% decreases in total N content of plants inoculated with Hup^- mutants compared to those inoculated with the wild type Hup^+ strain. Thus, these results support the theoretical expectations that Hup^+ strain would be superior to Hup^- strains. However, these comparisons also suffer from a few drawbacks: (i) in the comparison involving Hup^+ and Hup^- field isolates, the sample size of the Hup^- isolates was very small, and(ii) in the comparison involving the Hup^+ strains and Hup^- mutants, the isogenicity of the mutants and parent strains has not been established.

Rainbird et al (1983) studied the carbon and nitrogen economies of a single cultivar of cowpea nodulated with one high hydrogen-evolving strain and another low hydrogen-evolving strain of cowpea-Bradyrhizobium and estimated that the latter was more efficient (by 36%) in the utilization of carbon. However, the two symbioses did not differ in terms of N_2 fixation rate, total dry matter production and seed yield. The comparison made in this study was between two unrelated Bradyrhizobium strains nodulating a single cowpea cultivar and therefore these results may not be representative of a wider range of Bradyrhizobium strains and cowpea types.

The role of uptake hydrogenase in symbiosis has also been investigated with the fast-growing root nodule bacteria, for example R. leguminosarum which nodulates Pisum, Vicia, Lens and Lathyrus. Ruiz-Argueso et al (1978) observed no significant differences in dry weight

of plants nodulated with groups of high hydrogen-evolving and low hydrogen-evolving strains of R. leguminosarum. They also found that the hydrogenase activity in none of the strains in their study was sufficient to recycle all the hydrogen evolved by the nitrogenase system. Bethlenfalvay and Phillips (1979) surveyed five strains of R. leguminosarum for hydrogenase activity in pea nodules and found that the two strains which evolved relatively little hydrogen, had significantly higher rates of nitrogen reduction than the other three high hydrogen-evolving strains. Neither of the above studies provides good evidence either for or against the hypothesis to be tested, because in both studies very few (non-isogenic)strains have been compared.

In a survey of 108 isolates of R. leguminosarum, Nelson and Child (1981) observed no significant differences in dry weight and nitrogen content of Pisum sativum plants inoculated with Hup^+ and Hup^- strains. This experiment was conducted with large groups of strains under controlled environment conditions in growth chambers with 12 replicates and thus the experimental conditions in general were scientifically rigorous. These results suggest that the hydrogenase system in R. leguminosarum does not seem to provide significant advantage in the symbiotic performance with pea. The possible reasons why Hup^+ strains did not show superior performance in this study are: (i) none of the strains tested had sufficient hydrogenase activity to recycle all the hydrogen produced by nitrogenase, and thus the Hup^+

strains in this study had only intermediate levels of hydrogenase activity; and (ii) the hydrogenase negative strains had higher leghaemoglobin concentrations and thus appeared to have more nitrogen fixing tissues to compensate for high rates of evolution hydrogen evolution.

It has been discussed earlier that only a small percentage (5-10%) of fast-growing Rhizobium strains contained significant amount of uptake hydrogenase activity (see Table 5.1 in chapter 5). From the studies of Ruiz-Argueso et al (1978) and Nelson and Child (1981) mentioned above, it appears that even the small proportion of the strains having hydrogenase activity can not recycle all the hydrogen evolved during nitrogen reduction. Thus the levels of hydrogenase activity of the Hup^+ strains used in these studies were not comparable to those of any Hup^+ strains Bradyrhizobium. However, Nelson and Salminen (1982) recently found 4 isolates of R. leguminosarum that had hydrogen uptake activity similar or higher than those found in two Hup^+ B. japonicum strains. In comparison with Hup^- strains or other Hup^+ strains of R. leguminosarum with intermediate levels of hydrogenase activity, aerobically prepared bacteroids of these 4 superior Hup^+ strains, when incubated with H_2, formed significantly higher amount of ATP. Thus this result indicates that hydrogen oxidation is coupled to ATP formation in these Hup^+ isolates. Whether the ATP formation due to hydrogen oxidation in these strains results in increased dry matter of plants

was not tested in this study. These strains with high hydrogen uptake rates and isogenic Hup^- mutants derived from them should provide good experimental inoculant strains for the evaluation of the benefit of the H_2 recycling system in R. leguminosarum. Another interesting observation made in this study was that H_2 increased the optimal O_2 concentration for acetylene reduction in bacteroids from Hup^+ strains of R. leguminosarum by 1 kPa (0.01 atm). They suggested that protection against the presence of excessive O_2 may be the primary role of hydrogenase within the pea nodule. Hydrogen oxidation permits N_2 fixation at O_2 levels that are otherwise inhibitory in the absence of H_2.

Truelsen and Wyndaele (1984) also observed that in R. leguminosarum only a small minority of Hup^+ strains could recycle all the H_2 evolved during N_2 reduction. They used the term "recycle efficiency" (Ref) to denote the ability of a Hup^+ strain to recycle all the H_2 evolved during N_2 reduction and thus classified the hydrogenase positive strains into Hup^+Ref^+ and Hup^+Ref^- groups. However, they did not observe any significant effect of the Hup or Ref character on nitrogenase activity, dry weight and nitrogen content of plants in their comparison of 3 Hup^+Ref^+ and 19 Hup^+Ref^- strains with 4 Hup^- strains of R. leguminosarum.

From the experimental results involving comparison of Hup^+ and Hup^- strains for symbiotic performance as discussed above, it is not possible to arrive at any firm conclusion. However, some of the above results give some

indications that uptake hydrogenase may be beneficial in symbiosis involving Bradyrhizobium but it is of no consequence for fast growing Rhizobium strains.

6.3.2. Comparison of Hup^+ and Hup^- isogenic strains:

The availability of Hup^- mutants isogenic with the Hup^+ parents is necessary to demonstrate convincingly the benefits of H_2 recycling to the nitrogen fixation system in nodulated legumes. Mutants which do not revert may have multiple lesions or large deletions. Any difference between the symbiotic performance of such a non-revertible Hup^- mutant and its wild type Hup^+ parental strain may not be due to differences solely in hup genes. Lepo et al (1981) isolated 2 Hup^- mutants of B. japonicum which spontaneously reverted to the Hup^+ parental type at a frequency of 10^{-9} which is consistent with that of a single point mutation. The parent strain SR and revertible Hup^- mutants, PJ17 and PJ18 were used as inocula in an experiment with 10 replicates conducted under a controlled environment. Each replication in this experiment consisted of two plants grown in a single Leonard jar (a microbiologically contained plant growth vessel with a self-watering device). The soybean plants inoculated with the Hup^- mutant strains showed significant depression of growth as compared to those plants inoculated with the Hup^+ parent strain. Inoculation with the Hup^+ parent strain resulted in significantly higher plant dry weight and total nitrogen content than with the two Hup^- mutants (Table 6.2).

Table 6.2. Comparison of Hup^+ and Hup^- isogenic Rhizobium strains

Hup^+ strain and Hup^- revertible mutants compared	Experimental conditions	Host legume	%increase $*_1$ dry matter	%increase total N	Reference
Slow growing					
1. Hup^+ parent and Hup^- revertible mutants of B. japonicum	Growth cabinet	Soybean	17.0	36.0	Lepo et al (1981)
2. Two sets of Hup^- mutants and Hup^+ revertants of B. japonicum:	Growth cabinet	Soybean			Evans et al (1985)
(i) PJ17-19(Hup^-) vs PJ17-1-20 (Hup^+)			25.0	25.5	
(ii) PJ18 (Hup^-) vs PJ18-1 (Hup^+)			14.5	12.7	
Fast growing					
3. Three Hup^+ and six isogenic Hup^- mutant strains of R. leguminosarum	Growth cabinet	One vetch and three pea cultivars	NS*	NS	Cunninghum et al

* NS- non-significant; $*_1$-% increases in total dry matter and total N content of plants inoculated with Hup^+ strains compared to those inoculated with Hup^- isogenic strains.

The differences in the total dry weight and the total N content between the parent strain SR and the mutant PJ18 were larger than between SR and PJ17 (Table 6.2.1.). The bacteroids isolated from nodules formed by PJ17 and PJ18 showed no detectable hydrogen-uptake activity, whereas bacteroids formed by the parent strain showed high hydrogen uptake rate. Thus it has been shown that the hydrogenase system in the soybean-Bradyrhizobium symbiosis results in some improvement in the symbiotic performance. Although the improvement in the symbiotic performance of strain SR due to uptake hydrogenase was detectable, these differences were not very large, particularly with the mutant PJ17 (Table 6.2.1). It is also not known from this experiment if uptake hydrogenase in the Bradyrhizobium strain can lead to significant improvement in the seed yield of soybean plants.

The difference in the symbiotic performance of the Hup^- mutant PJ17 (Lepo et al, 1981) and its Hup^+ revertant PJ17-1 has been investigated by Drevon et al (1982). In an experiment where nodules were supplied with 20% or 40% O_2, the nodule-respiration of PJ17-1 was found to be 10% less than that of PJ17 nodules. However, acetylene reduction activity of the Hup^+ nodules (PJ17-1) was not significantly different from the Hup^- nodules (PJ17). Thus these results show that energy consumption through respiration in Hup^+ nodules is less than in Hup^- nodules. Whether this small saving of energy in respiration results in increased dry matter accumulation and higher yield of soybean plants has not

Table 6.2.1. Effect of Hup$^-$ mutants and the parent strain SR on total dry weight and N content of soybean plants (from Lepo *et al*, 1981)

Inoculating strain	Total dry weight (g)	Total N content (mg)
SR (Hup$^+$)	4.45 ± 0.19	88.5 ± 4.51
PJ17 (Hup$^-$)	4.17 ± 0.16	70.8 ± 4.56
PJ18 (Hup$^-$)	3.46 ± 0.19	58.6 ± 7.29

been reported in this study.

Evans et al (1985) compared the symbiotic performances of two Hup$^-$ mutants of B. japonicum with their respective Hup$^+$ revertants on soybean plants. The experiment was conducted in Leonard jars in the glasshouse. There were eight replications for each test and each observation was recorded on three plants grown for 40 days on a single pot. In the comparison involving strains PJ17-19 (Hup$^-$) and PJ17-1-20 (Hup$^+$), the use of the Hup$^+$ strain resulted in highly significant ($P \leq 0.01$) increases in the total plant material (25%) and in the total nitrogen (25.5%). The Hup$^+$ inoculant in the second comparison involving PJ18 (Hup$^-$) and PJ18-1 (Hup$^+$) also produced plants with significantly ($P \leq 0.05$) increased dry weight (14.5%) and nitrogen content (12.7%) (Table 6.2). In this experiment, isogenic Hup$^+$ and Hup$^-$ strains were compared in a controlled environment with eight replicates and data were statistically analysed. In this way the experimental material, the methods and the analyses of results satisfy the various conditions of the hypothesis under test. The experimental results provide evidence in favour of the hypothesis and support previous results of Lepo et al (1981).

In R. leguminosarum it has been shown earlier that the Hup determinants of strain 128C53 reside on a plasmid pRL6JI (Brewin et al, 1980). Kagan and Brewin (1985) isolated eight Hup$^-$ mutants from a streptomycin resistant derivative of the strain 128C53 through insertion of a transposon Tn5-mob into the symbiotic plasmid pRL6JI.

Physical and genetic evidence suggest that each of these Hup^- strains resulted from a single and independent insertion of Tn5-mob (see Fig 5.1 in chapter 5) and was isogenic with the parental Hup^+ strain. Recently, Cunningham et al (1985) compared six of these Hup^- mutants with the parental and two other isogenic Hup^+ strains for their symbiotic performance on a vetch and three pea cultivars. In every case at least one Hup^- mutant fixed as much N_2 as the isogenic Hup^+ strain. Measurements of C_2H_2 reduction, H_2 evolution, tritium incorporation and plant dry weight indicated that Hup activity in the Hup^+ strains in this study was not associated with increased N_2 fixation. Thus the results of this experiment are not in agreement with those in B. japonicum (Lepo et al, 1981; Evans et al, 1985). The symbiotic performance in this experiment was measured on four cultivars grown till flowering. Also the experiment was conducted apparently under proper conditions to detect small differences . However, under these conditions the Hup^+ strains were not found to be superior to the isogenic Hup^- mutants. This indicates that the Hup activity determined by pRL6JI is not a critical component of symbiotic efficiency. However, it is still possible that the Hup system in other strains of R. leguminosarum may be associated with increased N_2 fixation. It has also been reported that H_2 oxidation in strain 128C53 is not strongly coupled to ATP synthesis (Nelson and Salminen, 1982). Therefore, it would be desirable to use other Hup^+ strains of R. leguminosarum

such as those in which H_2 oxidation is strongly coupled to ATP synthesis and to compare them with their isogenic Hup^- mutants.

6.3.3. Comparison of Hup^- strains with genetically engineered Hup^+ derivatives:

The symbiotic plasmid pRL6JI of R. leguminosarum strain 128C53 is non-transmissible; however, when pRL6JI recombined with another Rhizobium plasmid, pVW5JI to form the recombinant symbiotic plasmid pIJ1008, it could be transferred into different Rhizobium strains (Brewin et al, 1982). Dejong et al (1982) transferred the recombinant plasmid pIJ1008 into the Hup^- field isolates, 300 and 3622 and obtained Hup^+ derivatives, 3960 and 3963 respectively. The two Hup^- strains and their Hup^+ derivatives were used to inoculate peas. The presence of the plasmid pIJ1008 in strains 3960 and 3963 resulted in significant increases in N_2 fixation, plant dry weight and total N content of plants relative to Hup^- field isolates, 300 and 3622 (Dejong et al, 1982; see Table 6.3.). The recombinant plasmid pIJ1008 contained genetic information for hydrogenase, nitrogenase, nodulation and other functions and in the strains carrying this plasmid the symbiotic N_2 fixation was enhanced. In the same study Dejong et al (1982) have shown that the transfer of a plasmid which encoded only Nif and Nod determinants, plus other genetic informations into the two recipient R. leguminosarum strains resulted in no increased N_2 fixation. Therefore, these authors

Table 6.3. Comparison of Hup^- strains with genetically engineered Hup^+ derivatives

Comparison of *Rhizobium* strains	Experimental conditions	Host legume	%increase dry matter*	%increase total N*	Reference
1. Two Hup^- strains of *R. leguminosarum* and their Hup^+ derivatives containing *hup* genes in recombinant symbiotic plasmid:					
(i) Strain 300 (Hup^-) vs strain 3960 (Hup^+ derivative)	Controlled environment	Pea	15.0	29.0	Dejong *et al* (1982)
(ii) Strain 3622 (Hup^-) vs strain 3963 (Hup^+ derivative)			87.5	111.0	
2. One Hup^- mutant strain of *R. japonicum* and a and a Hup^+ derivative obtained by recombinant techniques	Cylinder experiment under controlled environment	Soybean	9.0	11.0	Evans *et al* (1985)

*- % increases in total dry matter and total N content of plants inoculated with genetically engineered Hup^+ derivatives compared to those inoculated with Hup^- parental strains.

have indicated that the Hup$^+$ characteristic of pIJ1008 might possibly have caused the increased N_2 fixation. However, besides the Hup determinants, many other genetic determinants differ between pIJ1008 and the other symbiotic plasmids used in this study. This study shows that the transfer of an entire symbiotic plasmid (pIJ1008) between strains can bring about an improvement in symbiotic N_2 fixation, but these results do not provide specific evidence for the benefits of the Hup system in symbiotic N_2 fixation.

In B. japonicum, Evans et al (1985) obtained a Hup$^+$ derivative, PJ18nalHR from a Hup$^-$ mutant strain PJ18nal by recombinant techniques. The strain PJ18nal (Cantrell et al, 1983) is a spontaneous nalidixic acid derivative of the Hup$^+$ mutant strain PJ18 (Lepo et al, 1981). The Hup$^+$ strain PJ18nalHR was obtained by transferring the Hup cosmid pHU1 (tetracycline resistant) into PJ18nal by conjugation and subsequently from the transconjugants which were cultured in the absence of tetracycline, a tetracycline sensitive but hydrogenase positive recombinant strain was selected (Evans et al, 1985). The Hup$^+$ tetracycline sensitive could arise from a double cross over event between pHU1 and the homologous pJ18nal genome and subsequent loss of the cosmid by segreagtion in the absence of tetracycline. The strain PJ18nalHR was one such Hup$^+$, tetracycline sensitive isolate developed through recombination. Strain PJ18nal and PJ18nalHR should therefore be identical with the single exception of the mutated hup

gene in PJ18nal. Evans et al (1985) compared the symbiotic performance of PJ18nal (Hup^-) and the isogenic Hup^+ recombinant strain PJ18nalHR on soybean. They used large cylinders to grow inoculated soybeans to maturity in a controlled environment. Each treatment was replicated 10 times in this experiment and the data were statistically analysed.At harvest, plants inoculated with the Hup^+ PJ18nalHR, when compared with plants inoculated with Hup^- PJ18nal, produced significant increases in seed weight (5.2%), leaf weight (14%), root weight (16%), total dry weight (9%) and total N content (11%) [Table 6.3]. From these results it was concluded that the Hup^+ PJ18nalHR was superior to the Hup^- mutant PJ18nal as an inoculant for soybean. Thus this experiment provides strong evidence in favour of the hypothesis under test and supports the view that H_2 recycling in B. japonicum strains is beneficial to the soybean-Bradyrhizobium symbiosis. It would however be reassuring to recheck these growth experiments using a different revertible Hup^- mutation (eg PJ17) because PJ18 mutation has been shown to be a dominant Hup^- mutation (Haugland et al, 1984) and may therefore have exceptional phenotypic effects.

It has been shown (Lambert et al, 1985) that pLAFR1 cosmids pHU51 and pHU52 carrying hup DNA from B. japonicum carry sufficient information to confer hydrogenase activity to Hup^- field isolates of B. japonicum and also to Hup^- isolates of fast-growing strains, eg R. meliloti. In principle, the use of these hup DNA clones provides another opportunity for

comparing the symbiotic performance of isogenic Hup$^+$ and Hup$^-$ clones. However, the instability of the introduced pLAFR1 cosmids (with or without cloned *hup* genes) would add a high degree of uncertainly to the experimental results. No such plant growth experiments have yet been attempted, although no doubt they will be, as soon as cloned *hup* genes have been stably transferred into a previously Hup$^-$ *Rhizobium* strain.

6.4. Discussion:

The possible beneficial role of H_2 recling in legume-*Rhizobium* symbiosis has been experimentally tested mostly with either *B. japonicum* or *R. leguminosarum* strains as inoculants. Genetically unrelated Hup$^+$ and Hup$^-$ strains of *Rhizobium* have been compared as inoculants in most of these experiments. Although the results from this kind of experiment, comparing unrelated Hup$^+$and Hup$^-$ strains, do not provide any conclusive evidence, a few experiments with *B. japonicum* strains indicated a beneficial role of uptake hydrogenase system in symbiosis with soybean (Schubert *et al*, 1978; Carter *et al*, 1978; Albrecht *et al*, 1979; Zablotowicz *et al*, 1980; Hanus *et al*, 1981; Gibson *et al*, 1981). One similar experiment with mungbean *Bradyrhizobium* strains suggested the superiority of the Hup$^+$ strains as inoculants on mungbean (Pahwa and Dogra, 1981). However, in fast-growing *R. leguminosarum*,this kind of comparison involving genetically unrelated Hup$^+$ and Hup$^-$ strains did not show any advantage of hydrogen recycling in

symbiotic N_2 fixation in pea (Ruiz-Argueso et al, 1978; Bethlenfalvay and Phillips, 1979; Nelson and Child, 1981; Nelson and Salminen, 1982; Truelsen and Wyndaele, 1985).

In order to get convincing evidence about the possible beneficial role of uptake hydrogenase in symbiotic N_2 fixation, comparison must be made between isogenic Hup^+ and Hup^- strains. In B. japonicum, isogenic Hup^+ and Hup^- strains have been compared (Lepo et al, 1981; Evans et al, 1985) and the beneficial role of uptake hydrogenase in the soybean-B. japonicum symbiosis has been established. By comparing a Hup^- strain of B. japonicum with its Hup^+ derivative obtained through a recombinant technique, Evans et al (1985) demonstrated the superiority of Hup^+ strain over isogenic Hup^- strain as inoculants of soybean. Only one experiment has so far been reported in R. leguminosarum where isogenic Hup^+ and Hup^- strains have been compared for symbiotic performance on pea (Cunningham et al, 1985). The results of this experiment did not show any advantage in the Hup^+ strain 128C53 in their study. However, this result does not rule out the possible benefit of Hup system in other Hup^+ R. leguminosarum strains.

In a majority of Hup^+ R. leguminosarum strains the H_2 oxidation is not coupled to ATP formation (Nelson and Salminen, 1982). Ruiz-Argueso et al (1978) proposed that some deficiency might exist in a component of H_2 oxidation system in R. leguminosarum. Nelson and Salminen (1982) suggested that the major role of uptake

hydrogenase in R. leguminosurum might be primarily to protect nitrogenase function in the presence of excessive O_2. Although in a majority of the Hup^+ R. leguminosarum strains the H_2 oxidation was not coupled to ATP formation, there were few strains where H_2 oxidation was coupled to ATP formation similar to those of Hup^+ B.japonicum (Nelson and Salminen, 1982). Recently, Truelsen and Wyndaele (1985) used the term "Recycling efficiency"(Ref) to describe R. leguminosarum strains in which H_2 oxidation is coupled to ATP formation (Hup^+Ref^+). On the basis of these results it appears that only those Hup^+ strains where H_2 oxidation is strongly coupled to ATP formation and their isogenic Hup^- mutants can provide good experimental inoculant strains for the evaluation of H_2 recycling in pea-R. leguminosum symbiosis. The strain 128C53 and their isogenic Hup^- mutants as studied by Cunningham et al (1985) are not very ideal strains for such comparison; because it has been reported that H_2 oxidation in R. leguminosarum strain 128C53 is not stronly coupled to ATP synthesis (Nelson and Salminen, 1982) .

The Hup^+ R. leguminosarum strains are different from those of B. japonicum in that the Hup activity can not be determined on the basis of the ability to reduce methylene blue on agar plate or to grow chemolithotrophically. Interestingly, in a recent experiment Lambert et al (1985) have shown that cloned hup genes of B. japonicum in the cosmid pHU52 could confer the ability to reduce methylene blue and grow chemolithotrophically when

transferred to the R. leguminosarum strain 128C53. This indicates the possibility of constructing Hup$^+$ R. leguminosarum strains having hydrogen dependent chemoautotrophic growth through genetic engineering techniques. It would be interesting to know if such a genetically engineered strain can be a more efficient symbiont.

The occurence of hydrogenase in Rhizobium is very rare, particularly among the fast-growing strains. Only about 10% of R. leguminosarum strains and 25% of B. japonicum strains possess hydrogenase activity. However, among the free-living N_2 fixing organisms the occurence of hydrogenase is very common (Walker and Yates, 1978; Evans et al, 1981). The presence of hydrogenase in certain legume-Rhizobium symbioses could be just a relic from the evolution of nitrogen-fixing organisms (Drevon and Salsac, 1984b). Since strains of Rhizobium must carry out part of their life cycle as free-living organisms, it seems reasonable to postulate that Hup$^+$ strains have some advantage under certain ecological conditions. For example, Hup$^+$ strains of B. japonicum possess the capacity for maintaining themselves not only from organic carbon materials in tne soil but also from the use of the hydrogen produced from fermentative soil microorganisms. However, inside the nodules hydrogenase function may not be operative under environmental conditions such as under reduced availability of O_2 for respiration. Drevon and Salsac (1984a) have estimated the amount of ATP generated from oxidation of H_2 by

hydrogenase and from glycolysis of oxidative phosphorylation under reduced O_2 supply and observed that the oxidation of H_2 in Hup^+ nodules would generate less ATP than the oxidation of carbohydrate in Hup^- nodules with the same amount of O_2. Therefore, under the O_2 limitation condition inside root nodules, possession of hydrogenase by Rhizobium may not have an advantage. It may be due to some kind of selection pressure that caused Hup^- strains to be more abundant among various groups of Rhizobium (Evans et al, 1985).

In the case of temperate legumes,there is now good evidence for a rapidly responding system that regulates oxygen diffusion into nodules (Minchin et al, 1985). It is possible that this system has rendered hydrogenase inessential as a mechanism for consuming excess oxygen within the nodule. This in turn may account for the apparent lack of physiological importance for the hydrogenase system and the absence of the Hup^+ character from many field isolates of fast-growing Rhizobium strains.

6.5. Conclusions and perspectives:

From the review of the experiments as discussed above, the following conclusions could be made about the benefits of uptake hydrogenase in symbiosis:

(i) In soybean-B. japonicum symbiosis there are several conclusive observations which support the view that uptake hydrogenase is beneficial in this symbiosis. In the experiments where isogenic Hup^+ and

Hup$^-$ strains have been compared, 9-25% increase have been recorded in the dry matter content; also 25-36% in the total nitrogen content of plants inoculated with Hup$^+$ strains compared to plants inoculated with inoculated with isogenic Hup$^-$ strains (Lepo *et al*, 1981; Evans *et al*, 1985; see Tables 6.2 and 6.3).

(ii) In other bradyrhizobia such as cowpea-*Bradyrhizobium* and mungbean-*Bradyrhizobium* the evidence available so far indicates that possession of an uptake hydrogenase may be beneficial. In these *Bradyrhizobium* species more definitive experiments need to be conducted in which Hup$^-$ mutants and their Hup$^+$ revertants are compared as legume inoculants.

(iii) In pea-*R. leguminosarum* symbiosis also it has not been established whether uptake hydrogenase provides any advantage. The fact that in all Hup$^+$ *R. leguminosarum* stains H_2 oxidation is not strongly coupled to ATP formation, suggests that there must be proper choice of experimental strains in order to test the significance of uptake hydrogenase in pea-*Rhizobium* symbiosis. A Hup$^+$ strain in which H_2 oxidation is strongly coupled to ATP formation and its revertible Hup$^-$ mutant would be ideal for such comparison. More experiments with such strains need to be conducted in order to make conclusion about benefits of *hup* genes in *R. leguminosarum*.

Our appreciation of the potential significance of a functional H_2 oxidizing system in *Rhizobium* and factors affecting its expression within legume root

nodules has increased remarkably during the last few years. It has been established at least in B. japonicum that the possession of an uptake hydrogenase system is beneficial for symbiosis with soybean. Therefore, it would be desirable to incorporate this character into any otherwise good Hup$^-$ Bradyrhizobium and Rhizobium strains in order to produce superior strains. The progress that has been made in cloning and characterising hup genes should be useful in introducing the Hup character in Hup$^-$ strains through genetic engineering techniques.

The chemolithotrophic growth of B. japonicum may have special significance, because it may be important for growth and survival in the free-living state. The recent discovery that this character can be transferred to R. leguminosarum and R. meliloti (Lambert et al, 1985) shows the possibility that chemolithotrophy could be incorporated into fast-growing Rhizobium. It is possible that rhizobia capable of chemolithotrophic growth could have increased ablility to survive and multiply under awider range of soil environments.

Chapter 7

PROSPECTS FOR FUTURE EXPERIMENTAL WORK

It is now established that uptake hydrogenase plays a beneficial role in the soybean-*Bradyrhizobium* symbiosis. Although there is some evidence for a beneficial role of uptake hydrogenase in other legume-*Bradyrhizobium* symbiosis, more definitive experimental evidence would be required to confirm this conclusion. In the pea-*R. leguminosarum* symbiosis also, it is not very clear if uptake hydrogenase provides any advantage in N_2 fixation. Similarly in other fast-growing *Rhizobium* species, very little is known about the occurrence and the role of uptake hydrogenase.

In the light of the present status of knowledge concerning different aspects of uptake hydrogenase in root nodule bacteria, a few potential future experiments are given in outline and discussed below which would contribute to our understanding of the relationship between hydrogenase and energy-efficiency in the legume-*Rhizobium* symbiosis.

EXPERIMENT 1:

Title:- Symbiotic performance of Hup^+ and Hup^- isogenic *B. japonicum* strains on soybean.

Background:- It has been demonstrated earlier in two different experiments (Lepo *et al*, 1981, Evans *et al*, 1985, see chapter 6) that inoculation of soybean plants with Hup^+ *B. japonicum* strains produced greater dry matter and total nitrogen content of plants than those inoculated

with Hup$^-$ isogenic strains. However it would be desirableto compare different groups of Hup$^+$ and Hup$^-$ isogenic strains in future experiments, particularly because the Hup$^-$ mutation in the strain PJ18 used in these studies has been shown to be dominant and therefore may be having unusual negative pleiotropic effects.

Haugland et al (1984) developed 9 Hup$^-$ derivatives of Hup$^+$ B. japonicum strain 122DESnal by site-directed mutagenesis with Tn5 insertion. Tn5 insertions in different positions of the hup plasmid pHUI (Cantrell et al, 1983) were incorporated into corresponding homologous DNA of 122DESnal genome by marker exchange. Through DNA-DNA hybridization using ^{32}P-labelled pHU1::Tn5 as a hybridization probe to the EcoR1 digested total genomic DNA of these Hup$^-$ mutant strains, it was demonstrated that the Tn5 was inserted in an identical positions in the homologous DNA of these strains from pHU1. Therefore, these Hup$^-$ 122DESnal::Tn5 mutant strains should be isogenic to the Hup$^+$ parental strain 122DESnal. These Hup$^-$ strains can be compared with the Hup$^+$ parental strain for symbiotic performance on soybean. However, all these mutations are non-revertible being caused by Tn5 which may be having polar effects on other genes within the same transcriptional unit. Therefore, the revertible Hup$^-$ mutation pJ17 (Lepo et al, 1981) should also be included (The Hup$^-$ revertible mutant PJ17 was isolated from Hup$^+$ strain SR, which is a streptomycin resistant derivative of the strain 122DES).

Object of the experiment:- To compare the symbiotic

performance of Hup^+ B. japonicum strain 122DESnal with 10 Hup^- isogenic strains on soybean plants.

Materials and Methods:-

Inoculant strains:- R. japonicum strain 122DESnal and its 10 Hup^- derivatives containing Tn5 insertions on hup DNA (Haugland et al, 1984).

Plant tests:- Plant experiments could be conducted in two ways: i) in Leonard jars up to flowering, ii) in large concrete pots up to maturity.

i) Leonard jar experiment:- Plants will be grown in the Leonard jar as described by Evans et al. (1985). Soybean seeds will be surface sterilized with 2% sodium hypochloride and germinated inside sterile petri-dishes at 28°C. Germinated seeds will be transferred to sterile 1.2L Leonard jars and single plant will be grown in each jar. Each Leonard jar containing sterile washed river sand will be provided with 1L of nitrogen free nutrient solution. The solution will be replaced weekly. Each seedling in individual jar will be inoculated with a cell suspension containing about 7.0×10^8 cells per ml of the appropriate B. japonicum strain. Ten replicate cultures of each treatment will be arranged in a randomized block in a glass house, supplemented with light (16 h per day).

ii) Concrete pot experiment:- In order to evaluate the effect of uptake hydrogenase on symbiotic nitrogen fixation, it is desirable to grow the plants to maturity. Soybean seeds will be grown as single plants in large concrete pots (approximately 1 ft^3 in size) containing up to 4/5th, a sterilized mixture of 90% sand,

10% peat. This will be supplemented with 2g of K_2HPO_4. The plants will be watered twice a week with 1 L of nitrogen free nutrient solution per pot. Individual pot will be inoculated with a 5ml broth culture of the appropriate Rhizobium strain at the time of planting the seeds. The plants will be inoculated again after one week. Each treatment in this experiment will be replicated 10 times. Two additional control pots will be planted without inoculation. The plants will be grown in the glass house up to maturity.

Observations:- Plants will be harvested 40-50 days after inoculation. Observations will be recorded on C_2H_2 reduction activity and H_2 evolution by bacteroids, dry weight of nodules, shoots and total nitrogen content of plants.

Results and Interpretations:- The symbiotic performance of the Hup^+ parental strain 122DESnal is expected to be higher than that of the isogenic Hup^- 122DESnal::Tn5 mutants. If the results of the above experiment show signficantly higher performance of the Hup^+ strain than the Hup^- isogenic mutants, it would re-estabish the fact that uptake hydrogenase is beneficial in soybean-Bradyrhizobium symbiosis.

EXPERIMENT 2:

Title:- Effect of uptake hydrogenase activity determined by plasmid pRL6JI in R. leguminosarum on the symbiotic performance with pea having different levels of photosynthate supply.

Background:- Kagan and Brewin (1985) developed eight isogenic Hup$^-$ strains from the Hup$^+$ R. leguminosarum strain 3855 by mutagenesis with Tn5-mob. The Hup$^+$ phenotype of 3855 is determined by plasmid pRL6JI. This strain 3855 and 6 of its Hup$^-$ isogenic derivatives (Kagan and Brewin, 1985) have been tested for symbiotic performance on three commercial pea cultivars and one vetch line by Cunningham et al (1985). The results of this experiment showed no significant advantage of the Hup$^+$ parental strain over the Hup$^-$ isogenic strains in the symbiotic performance with the three pea cultivars and the vetch line used in this study. In another recent study Gordon et al (1985) demonstrated that nodule activity (respiration) is dependent on current photosynthesis in the leaves. It is possible that uptake hydrogenase may be beneficial for symbiotic N_2 fixation in root nodules having a limited supply of photosynthate. Therefore it would be desirable to test this possibility experimentally. The Hup$^+$ strain 3855 and its Hup$^-$ isogenic strains (Kagan and Brewin, 1985) may be used to inoculate two groups of pea plants, one group consisting of a commercial cultivar grown under normal growth conditions and the other group consisting of plants having reduced supply of photosynthates. A leafless pea line having only tendrils or a commercial pea cultivar grown under shorter light period than the normal 16 hours per day will presumably have a reduced supply of photosynthate. Whether uptake hydrogenase is beneficial for symbiotic N_2 fixation in root nodules having a limited supply of photosynthate, can

be tested by the following two experiments.

Experiment 'a', where the Hup^+ strain 3855 and its Hup^- isogenic mutants will be used to inoculate a normal commercial pea cultivar and an isogenic leafless pea line (Fig. 7.1) grown under normal 16 hours light conditions.

Experiment 'b', in which the Hup^+ strain 3855 and its Hup^- isogenic mutants will be used to inoculate several sets of a single commercial pea cultivar maintained at different light regimes.

Object of the experiments:- The aim of the above two experiments is to examine if the uptake hydrogenase activity determined by pRL6JI in strain 3855 has any beneficial effect with pea plants in which the supply of photosynthate is presumably limited.

Materials and Methods:-

Inoculant strain:- The Hup^+ strain 3855 and six Hup^- isogenic strains namely 520, 521, 522, 523, 524, and 525 (Kagan and Brewin, 1985, Cunningham et al, 1985).

Host cultivars:- One leafless pea line and an isogenic commercial pea cultivar will be used in experiment 'a'. Experiment 'b' will be conducted with a single commercial cultivar.

Plant tests:- Plant experiments will be conducted in 0.75L sterile Leonard jars under controlled environmental conditions in growth chambers as described by Cunningham et al (1985). Pea seeds will be surface sterilized and germinated under sterile conditions. Germinated seeds will be grown in Leonard jars as single plants. The experiment will contain at least 10 replications. All

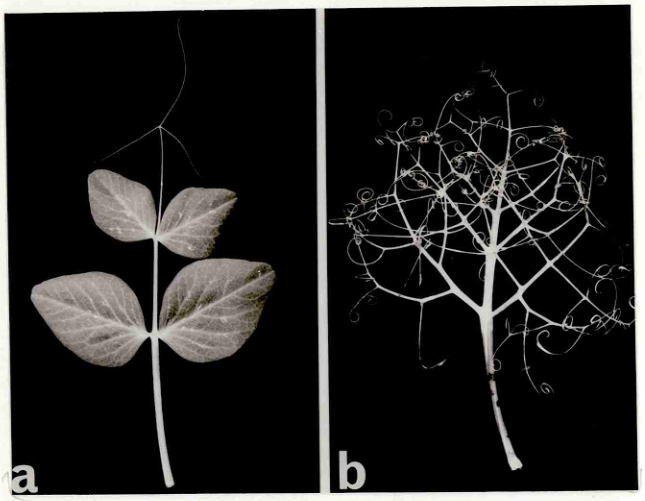


Fig 7.1. Photograph showing the conventional leaflet form (a) and the leafless form (b) of pea [Courtesy: Dr Peter Mathews, John Innes Institute]. The two forms are due to a single gene difference; the leaflet form (AfAf) and the leafless form (afaf). Isogenic pea lines of commercial cultivars with single gene difference for the leafless character are now available (Snoad and Hedley, 1981).

plants will be supplied with sterile N-free nutrient solution. Seedlings will be inoculated with a cell suspension of the appropriate Rhizobium strain.

The plants in experiment 'a' will be provided with light for 16 hours per day in the growth cabinet. Those in experiment 'b' will be grown in 3 sets of plants of a single cultivar provided with different light regimes, namely 16 hours, 13 hours and 10 hours per day.

Observations:- Plants will be grown for 40 days after germination. Observations will be recorded on C_2H_2 reduction activity and H_2 evolution by bacteroids, dry weight of nodules, shoots and roots and total nitrogen of plants.

Results and Interpretations:- It is difficult to predict the results from these experiments. It is possible that the Hup^+ parental strain 3855 might show better symbiotic performance than the Hup^- isogenic strain on the leafless pea line or on the commercial cultivar grown under shorter light hours per day. It would suggest that the uptake hydrogenase determined by pRL6JI is beneficial for symbiotic N_2 fixation only when the supply of photosynthate is the limiting factor in the root nodules.

EXPERIMENT 3:

Title:- Symbiotic performance of Hup^+ R. leguminosarum strain 128C30 and its Hup^- isogenic mutants in pea.

Background:- Recently Cunningham et al. (1985) have shown that the Hup activity of R. leguminosarum strain 3855 (128C53 str) determined by the plasmid pRL6JI does not

provide any advantage in symbiotic nitrogen fixation with pea. However, it is still possible that the Hup system in other strains of R. leguminosarum may be associated with increased N_2 fixation. Moreover it has been reported that H_2 oxidation in R. leguminosarum strain 128C53 is not strongly coupled to ATP synthesis (Nelson and Salminen, 1982). The strain 128C30 has been identified by Nelson and Salminen (1982) to possess a very high hydrogen uptake rate. In this strain hydrogen oxidation is also strongly coupled to ATP formation. This strain and Hup^- isogenic mutants derived from it, should provide good experimental inoculant strains for the evaluation of the benefits of the H_2 recycling system in R. leguminosarum.

Object of the experiment:- To compare the symbiotic performance of Hup^+ R. leguminosarum strain 128C30 with its Hup^- mutants on pea plants.

Materials and Methods:-

Rhizobium strain:- 128C30 (Nelson and Salminen, 1982).

Isolation of Hup^- isogenic mutants of 128C30:- Buchanan-Wollaston (1979) developed a generalized transduction system for phage mediated gene transfer between R. leguminosarum strains. By using the generalized transducing phage RL38, it is possible to transfer certain mutant alleles from one strain to another. Kagan and Brewin (1985) isolated eight Hup^- mutants from the Hup^+ R. leguminosarum strain 3855 using the transposon Tn5 mob. Any of these hup:Tn5 mob alleles from these Hup^- mutants will be transferred to the homologous position in the strain 128C30 through the generalized transduction using

phage RL38. A culture of strain 3855:hup:Tn5 mob and phage RL38 will be mixed with soft agar and spread as the top layer on agar plates. After two days of growth at 28°C the lysates from the Rhizobium cells will be sterilized with chloroform and will be irradiated with ultraviolet light at a dose rate of approximately $2Jm^{-2}s^{-1}$ (The transduction frequency increases by ultraviolet irradiation). Equal volumes of the strain 128C30 and the phage lysate will be mixed to give a multiplicity of infection of about one. The transduction mixture will be incubated at 28°C for three hours and then will be plated on the minimal medium. After 18 hours at room temperature the plates will be overlaid with 3ml soft agar containing kanamycin. These plates will be incubated for six days at 28°C. The kanamycin resistant colonies that will grow on these plates are expected to carry the Tn5:mob insertions; each kanamycin resistant clone should correspond to transduction of the hup::Tn5:mob mutation from the donor strain. However, to eliminate any spontaneous kanamycin resistant mutation, about 20 colonies will be purified and screened for Hup^- phenotype.

Random mutagenesis using Tn5:mob (Kagan and Brewin, 1985) is an alternative strategy to isolate Hup^- mutation in the strain 128C30.

Screening of Hup^- mutations:- At least three pea plants will be inoculated with each of the 20 mutant isolates. The bacteroids isolated from nodules of each plant will be screened for Hup phenotype using a method based on H_2 dependent methylene blue reduction recently developed by

Tichy and Lotz (1985). In this way it is expected to obtain a few Hup$^-$ isogenic mutants of strain 128C30.

Plant tests:- The Hup$^+$ strain 128C30 and its Hup$^-$ Tn5:mob insertion mutants will be used to inoculate plants of a commercial pea cultivar grown in Leonard jars as described in experiment 2 above.

Observations:- As in experiment 2 above.

Results and Interpretation:- The symbiotic performance of Hup$^+$ parental strain 128C30 is expected to be either significantly higher than or at par with the Hup$^-$ isogenic mutants. If the parental Hup$^+$ strain is found to be superior to the Hup$^-$ isogenic mutants it would suggest that the uptake hydrogenase activity of strain 128C30 is associated with increased N_2 fixation in symbiosis with pea.

EXPERIMENT 4

Title:- Symbiotic performance of Hup$^-$ B. japonicum and R. leguminosarum strains and their genetically engineered Hup$^+$ derivatives.

Background:- The development of superior mutant strains of Rhizobium is the ultimate goal of genetic research in Rhizobium. In order to develop such strains it would be desirable to incorporate important physiological characters such as hydrogenase activity into the commercial inoculant strains. Recently, Lambert et al. (1985) identified a 30 Kb region of hup DNA cloned in the plasmid pHU52 (see chapter 5) which contains genes for both the polypeptides of hydrogenase protein. This plasmid confers

Hup activity on several Hup$^-$ wild type B. japonicum strains. Also, transconjugants of Hup$^-$ B. japonicum and R. meliloti strains containing pHU52 showed hydrogen dependent methylene blue reduction and chemoautotrophic growth by virtue of the introduced genes. It would be interesting to study the symbiotic performance of the Hup$^+$ derivatives of Hup$^-$ B. japonicum and R. leguminosarum strains containing pHU52.

There may be some problems in studying the performance of such genetically engineered strains in nodules. The cloned hup DNA in pHU52 may be unstable in Rhizobium strains. Therefore further genetic manipulations may be necessary to produce stable genetically engineered Hup$^+$ strains. It would be desirable to stabilize the cosmid-borne hup genes by their integration into the host genome. Subcloning of hup genes in a relatively stable vector may be one way to stabilize the introduced Hup character in these strains. An alternative approach would be to introduce an essential gene into pHU52, so that it is stably maintained during replication. However, this kind of experiment aiming at stabilization of hup genes might require considerable effort and time.

Object of the experiment:- The aim of this short term experiment is to gain preliminary information about the performance of genetically engineered Hup$^+$ strains in nodules.

Materials and Methods:-

Inoculant strains:- Hup$^-$ B. japonicum and R. leguminosarum strains and their Hup$^+$ derivatives containing pHU52.

Plant tests:- B. japonicum and R. leguminosarum strains will be used to inoculate soybean and pea plants respectively. Soybean and pea plants will be grown in Leonard jars as described in experiments 1 and 2.

Observations:- The symbiotic performances of the Hup^- B. japonicum and R. leguminosarum strains and their Hup^+ derivatives containing pHU52 will be determined as described in experiments 1 and 2 above. The ex-nodule bacteria isolated from Hup^+ derivatives containing pHU52 will be tested for tetracycline resistance or hydrogenase activity in vitro.

Results and Interpretation:- If the bacteria isolated from Hup^+ nodules are found to be tetracycline resistant, it would suggest pHU52 was not lost from the strain inside the nodule. If however the ex-nodule bacteria are found to be tetracycline sensitive, indicating loss of pHU52, the interpretation of results will be difficult. It is expected that the Hup^+ strain containing pHU52 might show better symbiotic performance than their Hup^+ parental strain.

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